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Aggregation of teratocarcinoma stem cells with cleavage stage mouse embryos

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Aggregation of Teratocarcinoma Stem Cells with Cleavage Stage Mouse Embryos

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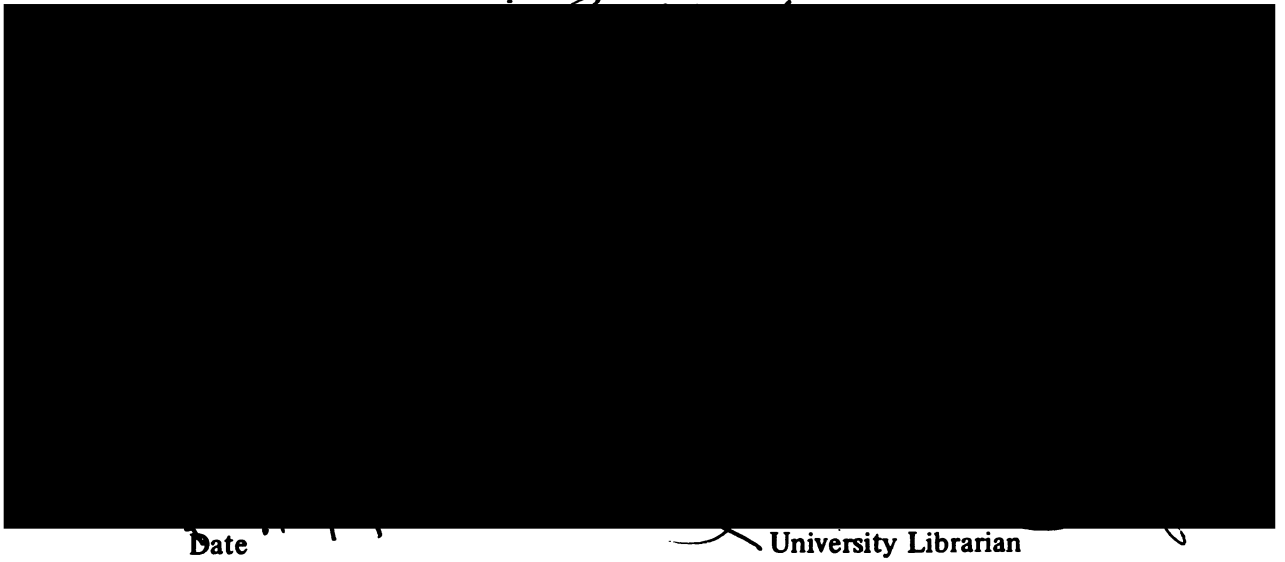

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ABSTRACT

Teratocarcinoma stem cells, also known as embryonal carcinoma cells, were aggregated with cleavage stage mouse embryos and their contribution to the composite embryos that subsequently developed was determined by glucose phosphate isomerase analysis. Three stages were examined: cultured aggregate blastocysts, 10-1/2 day midgestation fetuses, and live born animals.

When midgestation fetuses were examined, many were morphologically abnormal. This abnormal development is correlated with extensive participation by the embryonal carcinoma cells in the fetuses. This is true for all three of the cell lines studied, NG-2, PSA-1, and LT1-2D. Control experiments with embryo - embryo combinations suggest that a difference in developmental stage between the partners of an aggregate may adversely affect development. However, the data indicate that the abnormalities observed in the embryonal carcinoma - embryo chimeras are also the consequence of the inability of the embryonal carcinoma cells to independently support normal development and that the cells require association with a substantial number of host inner cell mass cells to form a normal fetus.

When aggregates were allowed to develop to term, the chimerism observed in the resulting animals was comparable both in frequency and in tissue distribution to that generally obtained in other studies using either the aggregation or blastocyst injection techniques.

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INTRODUCTION

Teratomas: Definition and Background

Teratomas are tumors that are characterized by a wide variety of well differentiated tissues representing all three germ layers. Derived from the Greek word teras, meaning "monster", the term teratoma aptly describes the often bizarre combination of well differentiated tissues commonly contained in these tumors. Teratomas are distinct from other tumors both in the broad heterogeneity of their tissues and in the complete foreignness of their tissues to the part of the body in which the tumors arise. They may contain only well differentiated tissues, in which case they are benign, or they may remain malignant by retaining a population of proliferating stem cells. Malignant teratomas are called teratocarcinomas to distinguish them from their benign counterparts although the term teratoma is still used loosely to refer to both types of tumor.

Teratomas were first described in humans (reviewed by Willis, 1967) in which they occur most often in the ovaries or testes but also occasionally in the anterior mediastinum, pre-sacral and coccygeal region, retroperitoneal region, and base of the skull. Ovarian and testicular teratomas usually appear during early adult life. Ovarian tumors are almost always benign and are typically cystic with very well differentiated tissues. In contrast, in adults, testicular teratomas are often highly malignant solid growths containing neoplastic stem cells as well as differentiated tissues. Most teratomas that occur in extragonadal sites are known to be present at birth and, with the exception

of those in the brain, they tend to be benign although they may sometimes cause problems when they impinge on adjacent structures. Tissues found in human teratomas include skin with hairs and sweat glands, respiratory epithelium, various glands, nervous tissue, bone, cartilage, adipose tissue, and undifferentiated embryonic mesenchyme.

A striking feature of well differentiated teratomas is the occurrence of well organized structures such as fingers and teeth. Observation of such structures in teratomas led to the popular belief that these tumors represent a suppressed fetus attached to an otherwise normal individual. There is no evidence to support this belief. No structure resembling a vertebral column has ever been positively identified and although some tissues are very well differentiated, they never form recognizable organs or body regions (Willis, 1958).

With the identification of certain strains of mice in which teratomas occur with relatively high frequency, followed by the development of two methods of experimentally inducing teratomas, these tumors became accessible both to morphological study and to experimental manipulation. What was formerly only an interesting, albeit provocative, phenomenon in human pathology developed into an important system in which to study both early development and the relationship between neoplastic and embryonic behavior.

Two strains of mice, 129 and LT, develop teratomas with high frequency. In the strain 129, approximately one percent of the male fetuses develop teratomatous foci in their seminiferous tubules between the fifteenth and seventeenth day of gestation (Stevens, 1962). These foci begin as small nests of cells that resemble undifferentiated, early

embryonic cells. As the foci expand, they sometimes organize into structures resembling the embryonic ectoderm of the mouse egg cylinder: columnar, ectoderm-like cells are arranged around a central cavity. The foci continue to enlarge, sometimes merging with each other, and subsequently develop into tumors. An increased incidence of testicular teratomas is found in mice of the substrain 129/Sv. Almost 10% of the males of this substrain contain testicular teratomas. This substrain was the result of backcrossing the gene steel (Sl^{J}) into the strain 129 (Stevens and Mackensen, 1961). A single gene mutation affecting tumor incidence and codominantly expressed was detected during breeding experiments. This mutation resulted in the subline 129/terSv in which 30% of the males develop testicular teratomas (Stevens, 1973).

Ovarian teratomas occur with high frequency in LT/ChReSv mice. Females of this strain begin to develop teratomas at around 30 days of age, and by 90 days of age about 50% have developed tumors. A morphological study of these tumors at different stages showed that they arise from oocytes which begin to develop parthenogenetically in the ovary. Beginning with cleavage to the two-cell stage, these oocytes appear to progress through the normal stages of development up to the early egg cylinder stage, at which time they become disorganized. From these disorganized structures, ovarian tumors arise. (Stevens and Varnum, 1974).

In 1959 Stevens found that testicular teratomas can be experimentally produced by transplanting 12- and 13-day fetal genital ridges from the strains 129, A/He, or their F-1 hybrids into histocompatible adult testes. Furthermore, it was found that teratomas of either sex can

be experimentally induced by transplanting normal embryos to extra-uterine sites such as the testes (Stevens, 1968) and the kidney capsule (Solter et al, 1970). If the transplanted embryos are eight days or older, only benign teratomas result (Damjanov et al, 1971). Transplantation of younger embryos results in both malignant teratocarcinomas and benign teratomas. (Stevens, 1968; Solter et al, 1970). The genetic background of the host animal also affects the incidence of malignant tumors. Embryo transfers to certain strains (C57BL and AKR) generally result only in benign teratomas whereas transfers to other strains (C3H, CBA, and A) result in malignant teratocarcinomas as well as benign teratomas (reviewed by Solter et al, 1975). Although the cause of this strain-related effect remains unknown, it has made possible the creation of malignant tumors from many strains of mice. Teratocarcinomas may be obtained from any strain by transplanting an embryo of that strain into the proper F1 host. Such a host would be the result of a cross between the strain of interest, making it histocompatible with the transferred embryo, and one of the strains associated with the development of malignant tumors (C3H, CBA, or A).

Attempts have been made to identify the cell of origin of teratocarcinomas by using experimentally induced tumors. Whether the tumors arise from embryonic stem cells or from primordial germ cells has been a longstanding controversy, manifest most clearly in the debate over classification systems of human tumors. One classification system groups teratocarcinomas together with seminomas under the heading "germ cell tumors". This is based on the argument that teratocarcinomas must be related to germ cells because they are most often found in the gonads.

An alternative classification system puts teratocarcinomas in a different category from seminomas, asserting that there is very little evidence that teratocarcinomas are derived directly from germ cells and that therefore there is no justification for grouping the two types of tumors together. (Melicow, 1965)

In an attempt to clarify this issue, Stevens (1967) transplanted genital ridges from animals homozygous for the gene steel, a mutation which dramatically decreases the number of germ cells, and found that very few developed teratomas. This result, combined with the observation that teratomatous foci initially appear completely enclosed in the seminiferous epithelium, led Stevens to suggest that testicular teratomas arise from primordial germ cells.

Although Stevens' experiments appear to suggest that testicular teratocarcinomas originate from primordial germ cells, studies involving embryo-derived teratocarcinomas, either experimentally induced or arising parthenogenetically in the ovary, suggest that teratocarcinomas originate from embryonic ectoderm. In morphological studies, normal developmental stages up to the egg cylinder stage are observed. These structures appear to become disorganized and overgrown by masses of embryonic ectoderm-like cells (Stevens, 1968; Stevens and Varnum, 1974). Transplantation of embryonic ectoderm alone results in teratocarcinomas whereas transplantation of extraembryonic parts yields no tumors (Diwan and Stevens, 1976; Solter and Damjanov, 1973).

In 1978, Mintz and her coworkers found that both sterile W/W embryos and sterile Sl^{J}/Sl^{J} embryos produce malignant teratocarcinomas when transplanted under the testis capsule. This demonstrates that early

embryonic cells, in the absence of germ cells, can give rise to teratocarcinomas, even though genital ridges without germ cells cannot. This is persuasive evidence that, at least in the case of embryo-derived teratocarcinomas, the tumors originate from disorganized masses of embryonic ectoderm cells rather than from definitive germ cells. In testicular teratocarcinomas as well, the tumors develop from nests of embryonic ectoderm-like cells, even though these cells arise from the germ cells without developing through any of the other normally intervening stages. It thus appears that although germ cells may give rise to teratomas in some cases, they do so by first giving rise to embryonic ectoderm-like cells. Thus, both the germ cell and embryonic cell theories of teratoma origin may be correct.

Embryonal Carcinoma Cells and Embryoid Bodies

The stem cells of teratomas and teratocarcinomas are called embryonal carcinoma cells. These are thought to be both the source of the differentiated tissues and the proliferating population of undifferentiated cells which maintains the malignancy of the tumor (Kleinsmith and Pierce, 1964). Populations of embryonal carcinoma cells may be maintained *in vivo* by sequentially passing the cells as solid or ascitic tumors in animals, or they may be established as cell lines *in vitro*.

Morphologically, embryonal carcinoma cells both *in vivo* (Stevens, 1959; Damjanov et al, 1971) and *in vitro* (Martin et al, 1977) resemble embryonic ectoderm cells of the early 5- to 7-day embryo. They are characterized by large nuclei containing two or three prominent nucleoli and by relatively undifferentiated cytoplasm with abundant free ribo-

somes, a few small mitochondria, occasional profiles of rough endoplasmic reticulum and Golgi apparatus, and a few membrane-bound dense bodies.

Embryonal carcinoma cells from certain tumors and cell lines form structures called embryoid bodies when grown in clumps in suspension either in vivo as ascitic tumors (Pierce and Dixon, 1959) or in vitro (Martin and Evans, 1975). Embryoid bodies begin as clumps of tightly associated, undifferentiated embryonal carcinoma cells. After a period of time in suspension they differentiate an outer layer of cells resembling endoderm. These are simple embryoid bodies. Certain tumor sublines and cell lines form cystic embryoid bodies: The appearance of endoderm is followed by the formation of a central cavity around which a layer of columnar epithelium develops. This cavity expands and occasionally, a third layer, ostensibly of mesoderm, forms between the outer endoderm layer and the inner columnar epithelium or ectoderm layer.

The development of embryoid bodies closely resembles that of the inner cell mass of mouse blastocysts shortly after implantation at 5 days of development and during subsequent development on days 6 and 7 (Martin et al, 1977). This comparison is even more striking when embryoid bodies are compared to isolated inner cell masses cultured in vitro (Wiley et al, 1978). Despite their close similarity certain differences do exist between embryoid bodies and isolated inner cell masses (reviewed by Martin et al, 1977): Inner cell masses form mostly visceral endoderm whereas embryoid bodies form both visceral and parietal endoderm; inner cell masses form what appears to be extra-embryonic ectoderm whereas embryoid bodies never form extra-embryonic ectoderm

(that inner cell masses form extra-embryonic ectoderm is surprising because extra-embryonic ectoderm is thought to be a trophectoderm derivative [reviewed by Gardner and Papaioannou, 1975]); inner cell masses rarely attach to a tissue culture substratum after endoderm formation, whereas embryoid bodies will attach to a substratum at all stages of development; all inner cell mass cells form a columnar epithelium around a centrally placed cavity, whereas in some embryoid bodies the cavity forms eccentrically in which case the columnar epithelium fails to surround the cavity completely and some cells remain undifferentiated. Both embryoid bodies and inner cell masses cultured in vitro appear to form mesoderm, however the mechanism by which they do so is unclear and appears to differ from the process in vivo in which cells from the primitive streak migrate to form mesoderm between the ectoderm and endoderm.

Embryonal carcinoma cells in embryoid bodies also differ from normal embryo cells in the length of their cell cycle and in the timing of developmental events. Isolated inner cell masses in culture take approximately 24 hours to form endoderm, another 24 hours to form a cavity, and two to three days more to expand and form mesoderm (Hogan and Tilly, 1978). This is comparable to the time required for these events in vivo although mesoderm formation and further development progresses much more rapidly in vivo, taking only 12 to 24 hours rather than days (Rugh, 1968). In contrast, although embryoid bodies will form endoderm within 24 hours, an additional three to four days are required for cavity formation and another four to six days are required for full expansion and mesoderm formation (Martin et al, 1977). Thus embryoid

bodies require a period of time approximately twice as long as the period required by inner cell masses in vitro and three times as long as the period required by inner cell masses in vivo to develop through the same series of events. In terms of the cell cycle, embryonal carcinoma cell cycle times are much longer than embryonic cell cycle times at this stage. Cell cycle times of cultured embryonal carcinoma cells range from twelve to twenty-one hours (Martin and Evans, 1975; Papaioannou et al, 1978) in contrast with embryonic cell cycle times which have been reported to average as long as eight hours and as short as five hours during this period (see Poelmann, 1980 for review).

Despite the differences discussed above, embryonal carcinoma cells are very similar to early embryonic ectoderm cells both in their morphology and in their behavior. The few biochemical and immunological studies that have been completed on embryonal carcinoma cells thus far support this comparison. Alkaline phosphatase, an enzyme whose role in development is unknown but whose activity level changes dramatically during embryogenesis, is localized early in development to the inner cell mass and later to the embryonic ectoderm. In agreement with the general comparison between embryonic ectoderm and embryonal carcinoma cells, alkaline phosphatase activity is also found localized to the embryonal carcinoma cells of embryoid bodies, and, if present, is not apparent in the outer layer of differentiated endoderm (Damjanov et al, 1971; Bernstine et al, 1973).

Plasminogen activator synthesis in early embryogenesis is associated with trophoblast, parietal and visceral endoderm, and extra-embryonic mesoderm (Strickland et al, 1976; Bode and Dziadek, 1979).

Consistent with the analogy to early embryos, plasminogen activator synthesis in teratocarcinoma embryoid bodies is also associated with the presence of endoderm (Linney and Levinson, 1977). Two types of plasminogen activator have been identified in the embryo. One is associated with parietal endoderm, the other is associated with visceral endoderm and extra-embryonic mesoderm. When F9 embryonal carcinoma cells are induced to differentiate endoderm, they also begin to secrete the parietal endoderm type of plasminogen activator (Marotti et al, 1982).

Examination of chondroitin sulphate synthesis in pre-implantation embryos and embryonal carcinoma cells reveals that the teratocarcinoma synthetic patterns differ significantly from patterns of pre-implantation embryos, again, consistent with the comparison to embryonic ectoderm cells of the post-implantation embryo (Cantor et al, 1976).

In comparisons of embryonal carcinoma cells with mouse embryos using one-dimensional SDS-polyacrylamide slab gel electrophoresis with autoradiography (Martin et al, 1978), embryonal carcinoma cells were found to exhibit a pattern of protein synthesis similar to that of embryonic ectoderm. This pattern is not shared by other cultured cell types or embryonic cells and includes at least one prominent 55,000-dalton protein not detectable at earlier stages. Studies examining two-dimensional electrophoretic patterns of protein synthesis generally confirm the lack of similarity between the synthetic patterns of embryonal carcinoma cells and pre-implantation embryos (Dewey et al, 1978) and reiterate the similarity between embryonal carcinoma cells and embryonic ectoderm cells as represented by cultured ICM cells (Howe et al, 1980).

There have been several immunological studies which examine the cell surface antigens of embryonal carcinoma cells (reviewed by Hogan, 1977; Martin, 1978). These involve sera raised against embryonal carcinoma cells both in syngeneic mice (Artzt et al, 1973) and in rabbits (Stern et al, 1975; Kemler et al, 1977). Sera have also been raised against embryonal carcinoma cells using the monoclonal antibody technique (Solter and Knowles, 1978). These sera recognize a variety of adult tissues and embryonic stages including early cleavage stages. It is not known how strictly stage specific these antigens are, and no correlation has been made conclusively between embryonal carcinoma cells and any particular embryonic stage using these sera. For example, although Fab fragments of IgG raised against embryonal carcinoma cells in rabbits can reversibly inhibit cleavage stage embryos and morulae from compacting (Kemler et al, 1977), the antigen which is being blocked by the Fab fragments may also be present on the embryonic ectoderm of later stages. Thus, while it is apparent that embryonal carcinoma cells share specific cell surface antigens with early embryos, the stage specificity of these antigens remains unclear.

In Vitro Cell Culture of Embryonal Carcinoma Cells

As mentioned earlier, embryonal carcinoma cells can be isolated from solid or ascitic tumors and established in cell culture. Many cell lines have been established (reviewed by Martin, 1975, 1978; Graham, 1977). These cell lines vary considerably in their ability to differentiate *in vivo*: some cell lines form tumors with many differentiated tissues, whereas others form tumors with very few, if any, differen-

tiated tissues. There is also considerable variation in their ability to differentiate in culture: some cell lines form cystic embryoid bodies whereas others do not. This is in part a reflection of the tumors from which they were isolated. Some tumors lose their ability to differentiate when they are serially retransplanted in mice. These tumors become dominated by undifferentiated embryonal carcinoma cells and differentiate few, if any, tissues. Cell lines established from such "nullipotent" tumors are, themselves, nullipotent (Martin and Evans, 1975).

Culture history may also affect differentiative capacity. For example, several embryonal carcinoma cell lines are maintained on feeder cells, that is, they are co-cultured with fibroblastic cells whose ability to divide has been inhibited by treatment with mitomycin-C or X-irradiation. When such embryonal carcinoma cells are adapted for growth without feeder fibroblasts, they lose the ability to differentiate embryoid bodies in culture (Hogan, 1976). This does not mean that cell lines that are initially isolated from tumors and established in cell culture without feeders will not differentiate. Some feeder independent lines have been found to differentiate both when injected into mice and when cultured in vitro (Nicolas et al, 1976; Mintz and Cronmiller, 1981).

Even among cell lines that form embryoid bodies, there is considerable variation. Certain cell lines are able to differentiate cystic embryoid bodies with expanded central cavities, whereas other cell lines form only simple embryoid bodies. Nullipotential cell lines form clumps of cells but do not develop endoderm.

Since embryonal carcinoma cells are easily manipulated *in vitro* and are available in large quantities for biochemical work, they are commonly used as a model system in which to study early embryonic stages and differentiative events. For example, they have been used extensively to raise antisera which will react against embryonic cell surface molecules as well as to identify potential embryonic cell surface lectins (Grabel et al, 1979). The appearance of new cytoskeletal elements during endoderm differentiation has been examined in embryoid bodies (Kemler et al, 1981; Oshima, 1981). Substances that are synthesized during early embryonic development, such as alkaline phosphatase, Reichert's membrane, and alpha-fetoprotein, have also been studied using embryonal carcinoma cells (reviewed by Hogan, 1977). Certain embryonal carcinoma cells can be used as a model system for the study of X-inactivation. Experiments show that during differentiation these female embryonal carcinoma cells decrease their level of activity of X-linked gene products by 50% to the level found in XO embryonal carcinoma cells (Martin et al, 1978).

These studies depend on the strength of the analogy between embryonal carcinoma cells and early embryo cells. A contrasting approach is to use teratocarcinomas as a system in which to study neoplastic growth resulting from a disturbance in normal development. The growth of tumors from parthenogenetically activated oocytes in the ovary and from ectopically placed embryos in the kidney capsule or testis suggests that teratocarcinomas arise from embryo cells which find themselves in a foreign environment, i.e. an ovary, testis, or kidney capsule. Their neoplastic behavior may not involve transformation *per se* but simply may

be due to the close relationship between neoplasia and the embryonic state. It has been suggested that embryonal carcinoma cells are entirely normal embryonic cells which, because they are in an inappropriate environment and lack the proper developmental cues, continue to proliferate and differentiate haphazardly (Dominis et al, 1975; Damjanov and Solter, 1975). This is not a new concept. Soon after Spemann's theories regarding embryonic induction became known, it was suggested that teratomas could be the product of embryonic primordia that had escaped the control of the primary organizer (reviewed by Needham, 1942; Willis, 1958). This does not mean that teratocarcinomas are unrelated to other types of cancer in general, although it is certainly possible that teratocarcinomas may be a unique and atypical case of abnormal development involving no malignant transformation. As Nicholson wrote in 1929, "...the cause of cancer is known today as precisely as that of physiological growth, and ... the last word will not be spoken on the one an instant sooner than on the other."

Many investigators believe that teratocarcinomas are, in fact, neoplastically transformed and have used teratocarcinomas to underline the developmental aspects of neoplasia (Pierce, 1967) and to suggest that embryonic stem cells are the most logical targets of carcinogenesis (Pierce, 1974; Mintz, 1977). Furthermore, the example of teratocarcinomas has been used to support the theory that carcinogenesis is a reversible, epigenetic event (Pierce, 1967) or, at the very least, does not necessarily involve the loss of normal developmental capabilities (Mintz, 1977). As support for this hypothesis, proponents have cited the fact that embryonal carcinoma cells can be re-incorporated into the

normal pattern of development and can contribute to the development of a healthy mouse when placed in an appropriate embryonic environment. Notwithstanding the possibility that teratocarcinomas are a unique exception in the phenomenon of neoplasia, embryonal carcinoma cells clearly display neoplastic behavior and just as clearly are able, given the appropriate circumstances, to grow and differentiate in normal developmental patterns (Illmensee, 1978). They are thus an example of how neoplastic behavior, if not the neoplastic state itself, may be brought under normal developmental control.

Embryonal Carcinoma Cell - Embryo Chimeras

Embryonal carcinoma cells were first shown to participate in completely normal development by Brinster (1974, 1975). Using a micromanipulator, he injected two to four embryonal carcinoma cells isolated from embryoid bodies of a 129/SvSl (agouti) embryo-derived tumor (OTT 6050) into a mouse blastocyst. Following transfer of the injected blastocysts into pseudopregnant females, 60 offspring were reared to adulthood. One had agouti hairs in his otherwise albino coat. These agouti hairs could have been derived only from the injected embryonal carcinoma cells since the host blastocyst was genetically albino. Because there were no other known genetic markers to distinguish the cells of the random bred host blastocyst from the injected embryonal carcinoma cells, it was not possible to determine whether additional tissues had been formed by embryonal carcinoma cell derivatives in this animal.

Use of inbred recipient blastocysts, either WH or C57BL, made

possible a more thorough analysis of embryonal carcinoma cell contribution primarily by use of glucose phosphate isomerase (GPI) assays (Mintz and Illmensee, 1975; Illmensee and Mintz, 1976). Mintz and Illmensee, also using embryonal carcinoma cells from embryoid bodies of OTT 6050, injected blastocysts with either five cells or one cell. Of the mice that developed from these blastocysts, 13.9% (13/93) and 25.3% (18/71), respectively, were chimeric, that is, contained derivatives of both cell types, the host blastocyst cells and the injected embryonal carcinoma cells, in their tissues (Illmensee, 1978). Although chimerism was sporadic, several mice having only one or two tissues containing derivatives of the embryonal carcinoma cells, at least one mouse was extensively chimeric. In this mouse, embryonal carcinoma cells contributed heavily to all tissues, in some cases forming as much as 90% of the tissue as estimated from GPI enzyme analysis (Mintz and Cronmiller, 1978). In this series of experiments, embryonal carcinoma cell contributions were made to a wide variety of tissues including blood, brain, spleen, heart, liver, lungs, and reproductive tract. In two cases, breeding tests demonstrated the formation of functional sperm by embryonal carcinoma cells.

These results aroused a great deal of interest not only because injection of embryonal carcinoma cells into blastocysts offers a system in which to study the return of a neoplastic cell to a normal pattern of growth, but also because, potentially, it offers a powerful tool for manipulating the genetic composition of mice. It is possible to modify the genetic constitution of embryonal carcinoma cells in culture by mutagenesis followed by appropriate selection procedures, by fusion with

another cell or a microcell containing extrinsic genetic material, or by DNA-mediated transformation. Hypothetically, genetic alterations could be introduced into the tissues of a mouse by incorporating such altered embryonal carcinoma cells into a blastocyst. Using this method, the behavior of specifically modified genes could be studied in the context of an *in vivo* environment, the body of a mouse. Moreover, if these modifications corresponded to genetic lesions known to be responsible for specific human diseases, chimeric mice containing such lesions would provide useful animal models for studying these diseases. Such animal models would be particularly valuable for studying diseases for which no analogous animal disease exists. The mosaic distribution of embryonal carcinoma cell derivatives among the various tissues of chimeric mice might make possible the study of tissue specific effects. Germ line contribution by the embryonal carcinoma cells would make available, in successive generations, mice with the genetic modification in all of their cells.

Since the above stategy was conceived, several other methods for directly manipulating the genetic composition of mice have been explored. These include replacing the nucleus of a developing oocyte with a foreign nucleus (nuclear transplantation), injecting foreign DNA into the pronucleus of a developing oocyte, and injecting stem cells into fetal or adult mice. Each method involves a distinct combination of technical problems and theoretical advantages.

Nuclear transplantation has the advantage of modifying all of the cells in the embryo at once (Illmensee and Hoppe, 1981). If the nuclei of genetically modified embryonal carcinoma cells, for example, prove

capable of directing development from the one-cell stage, this may be the method of choice for creating new, genetically altered mouse strains. It is possible, however, that modified embryonal carcinoma cell nuclei will not be able to independently support development without first being cycled through development in the company of other mouse embryo cells. If this is the case, then nuclear transplantations will not be particularly useful for creating new mouse strains until a source of readily accessible cells (or nuclei) with full developmental capabilities is found.

An exciting amount of progress has been made recently in incorporating foreign DNA into the chromatin of developing mouse oocytes. Expression of this DNA has been detected in a couple of cases (Wagner et al, 1981; Brinster et al, 1981) and the foreign DNA has passed through the germ line to offspring in at least one case (Gordon, 1981). In all cases, however, there appears to have been extensive multiplication and a varying degree of rearrangement of the injected genes. In addition, these genes are randomly inserted and in one case appear to have interfered with the meiotic development of germ cells in the host animal (Gordon, 1981). Thus, while injection of DNA into oocytes appears to be a feasible method for inserting new genes into mice, several complications regarding the interactions of the exogenous genes with the endogenous genome and the preservation of the integrity and function of the injected genes need to be resolved.

Both of the above methods affect all of the cells of the developing animal at once. The opposite end of the spectrum is to change only one cell population using foreign stem cells. This has been done <u>in utero</u> by

injecting hematopoietic stem cells into the fetal placenta (Fleischman and Mintz, 1979). In adults, methotrexate resistant bone marrow cells have been injected into irradiated mice subsequently treated with methotrexate (Cline et, 1980) and fetal trisomy 12 or 19 hemopoietic stem cells have been shown to restore hemopoiesis in irradiated mice (Herbst et al, 1981). While these experiments appear to have successfully altered endogenous stem cell populations, the method is severely limited to tissues with easily accessible stem cell populations and requires that the host animal cells be put at a selective disadvantage. Although this method will undoubtedly have clinically relevant applications, its usefulness for experimentally manipulating gene expression in mice would seem to be limited.

The use of teratocarcinoma cells in chimeric mice has several advantages as well as disadvantages when compared with the above techniques. Although only a portion of the cells in an animal are initially replaced in chimeric mice, the developmental capabilities demanded of the altered cell are not as stringent as in the case of nuclear transplantations. It is possible that a cell not able to support full development on its own, perhaps due to mutagenesis, will be rescued by surrounding embryonic cells. Furthermore, such a cell, once exposed to the full developmental program, might eventually be able to contribute to the germ line. In contrast to the injection of DNA into oocytes, the selection for specific DNA alterations in embryonal carcinoma cells is done before mouse embryos are involved, in the more permissive environment afforded by cell culture. Consequently, genetic modifications are more specific and may involve alteration of endogenous gene se-

quences as well as addition of exogenous DNA, although this may necessitate exposing the entire genome to a mutagenic agent. As with the injection of foreign stem cells, the use of embryonal carcinoma cells in chimeric mice results in a mosaic rather than a homogeneous distribution of altered cells in the resulting animal. Although the tissue distribution of embryonal carcinoma cells in chimeric animals is fortuitous and experimentally uncontrollable, the range of tissues in which they can occur is much broader than that possible with the stem cell technique. Thus, the potential exists for examining a wide variety of tissue effects, even though significant technical problems remain to be solved before this is realized.

It is clear from the above discussion that the incorporation of modified embryonal carcinoma cells into chimeric mice potentially offers a significant number of advantages and merits further development as a method for altering the genetic composition of mice. Four sets of experiments have indicated that such incorporation of genetically modified embryonal carcinoma cells into chimeric mice is feasible.

Dewey et al (1977) injected hypoxanthine phosphoribosyltransferase (HPRT) deficient embryonal carcinoma cells into blastocysts. These cells were derived from a pluripotent embryonal carcinoma cell line (PSA-1) by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine followed by selection for resistance to the purine base analog, 6-thioguanine. Of the forty-four live mice obtained from injected blastocysts, twelve were autopsied, and nine of the twelve had tissues containing HPRT deficient embryonal carcinoma cell derivatives. Of the nine chimeric mice, three mice contained nine or more tissues containing

major contributions, as much as 50%, from the injected embryonal carcinoma cells. The remaining chimeras, however, had extremely small embryonal carcinoma cell contributions which were generally limited to one tissue.

Mutant genes in mitochondrial DNA were introduced into mice by Watanabe et al (1978). They injected cultured embryonal carcinoma cells that had been fused with cytoplasts of chloramphenicol resistant melanoma cells. Twenty-five mice were obtained from injected blastocysts. Five of the six mice autopsied contained mutant embryonal carcinoma cell contributions: two in 10-15% of their spleens, two in 10-15% of their brains, and one in 10-15% each of his muscle and brain.

Illmensee and his co-workers (1978, 1979) have reported two cases of incorporation of embryonal carcinoma cells containing xenogeneic chromosomes. In their first set of experiments they injected embryonal carcinoma cells that had been fused with human diploid fibrosarcoma cells. Forty-nine mice were obtained from injected blastocysts. Of these, two mice contained both coat and internal tissue contributions from the hybrid embryonal carcinoma cells. Expression of human-specific enzyme (galactokinase), however, was limited to ambiguously low levels of activity in the heart of one and in the kidney of another chimeric mouse. In their second set of experiments they injected embryonal carcinoma cells that had been fused with rat hepatoma cells and obtained more extensive xenogeneic gene expression. Of the sixty-two mice developed from injected blastocysts, three showed hybrid embryonal carcinoma cell participation in their tissues. Nine different rat-specific enzyme variants were detected in these tissues, perhaps re-

flecting the larger number of rat chromosomes retained in the mouse-rat hybrid embryonal carcinoma cells relative to the number of human chromosomes retained in the mouse-human hybrids.

While the positive results of the above experiments are encouraging, careful consideration reveals a number of difficulties. To be useful, chimeric mice should be obtainable in reasonably large numbers and should each, as consistently as possible, contain a moderately high level of chimerism in their tissues. High levels of chimerism, in even a few mice, would increase the probability of germ line contribution by the embryonal carcinoma cells. Germ line contribution is particularly desirable because it would make available mice containing the genetic alteration in all of their tissues and, furthermore, would circumvent the complicated and expensive procedures involved in generating chimeric mice.

In the experiments cited above, involving genetically altered cells cultured in vitro, the number of chimeric mice obtained was low. Although occasionally a mouse contained moderately high levels of embryonal carcinoma cell participation in its tissues, chimerism was sporadic. As suggested by these experiments, usable numbers of chimeric mice containing large populations of mutant embryonal carcinoma cells in their tissues are not dependably obtainable at this time. Germ line contribution has never been achieved using embryonal carcinoma cells which have been genetically altered in vitro. A recent report by Stewart and Mintz (1981) describes germ line incorporation by cells from a diploid embryonal carcinoma cell line, METT-1. This is noteworthy since previously the only reports of germ line incorporation were in experi-

ments using tumor-derived cells maintained *in vivo* (reviewed by Illmensee, 1978).

One factor which may affect the level of chimerism in mice developing from injected blastocysts is the developmental potential of the cell line that is used. As discussed earlier, there is great variability among embryonal carcinoma cell lines. They differ in the extent to which they will form differentiated cell types both *in vivo* and *in vitro*, in their culture requirements, and in their karyotypes. These differences are reflected in their ability to contribute to the tissues of a chimeric mouse (Papaioannou et al, 1978; reviewed by Papaioannou, 1979). Generally, those cell lines which differentiate well *in vitro*, which form large, well differentiated tumors, and which form cystic embryoid bodies, also develop well in chimeric mice. Another factor which may affect the ability of embryonal carcinoma cells to contribute to chimeric mice is their karyotypic abnormality. Germ line incorporation in particular, may require a normal karyotype. With these points in mind, it is encouraging that a diploid cell line with extensive developmental potential has been reported by Stewart and Mintz (1981). These cells were able to contribute broadly to the tissues of 41/312 (13%) experimental animals including one case of germ line contribution. It is hoped that these cells will retain their developmental potential once they are subjected to genetic modification.

A variety of other factors might hinder the incorporation of injected embryonal carcinoma cells into normal blastocysts. For example, the cell cycle time of embryonal carcinoma cells is much longer than that of the embryonic cells of the developing blastocyst: twelve to

twenty-one hours compared to an average of five to eight hours in embryos. Illmensee and Mintz (1976) suggest that there is a tendency toward delayed embryonal carcinoma cell integration into the blastocyst perhaps due to an initial difference in cell adhesiveness between the donor and host cells. They also suggest that the number of cells injected affects the efficiency of their integration.

Specific Aims and Rationale of This Study

This study was initially undertaken to assess the ability of embryonal carcinoma cells to integrate into the normal program of development. It had been shown that embryonal carcinoma cells could form functional germ cells in a chimeric mouse and that, in conjunction with the genome of the other parent, their genome could direct the normal development of the tissues of an entire mouse (Mintz and Illmensee, 1975). It was not clear, however, whether orderly expression of this totipotency required prior cycling of the embryonal carcinoma cells through normal embryonic development in close association with other embryonic cells. Morphological (Stevens, 1975; Martin et al, 1977) and biochemical (Martin et al, 1978) data suggested that embryonal carcinoma cells correspond to embryonic ectoderm. Since embryonic ectoderm is unable to differentiate trophoblast which is required for a successful relationship with the uterine environment (Rossant, 1975), it was thought that embryonal carcinoma cells might need other embryo cells simply to provide trophoblast. To test this possibility, the initial intent was to minimize the contribution of host embryonic cells to the inner cell mass, limiting them, if possible, to the formation of tropho-

blast and leaving the associated embryonal carcinoma cells to form the major portion of the inner cell mass.

The above strategy would also increase the incorporation of embryonal carcinoma cells into chimeric mice. As discussed previously, earlier experiments had achieved only sporadic chimerism and very rare germ line incorporation. Considering their potential usefulness, it seemed appropriate to attempt to generate animals composed largely of mutant embryonal carcinoma cells.

Aggregation of cleavage stage embryos had been used extensively for producing chimeric mice (McLaren, 1976) and it was thought that this method might be adapted to produce chimeric mice with embryonal carcinoma cells. Studies with normal embryonic cells had established that inner cell mass cells could successfully aggregate with cleavage stage blastomeres to form normal, chimeric, mid-gestation embryos (Rossant, 1975). It seemed possible, therefore, that embryonal carcinoma cells could also aggregate with cleavage stage embryos despite the substantial difference in their developmental stages.

Embryonal carcinoma cells aggregated with cleavage stage embryos would be in the embryonic environment for at least 24 hours longer than embryonal carcinoma cells injected into blastocysts. This longer period of association might allow them to synchronize more effectively with the embryo, by giving them more time to adjust their cell cycle time, alter metabolic pathways, and so forth. Furthermore, by arranging the normal embryonic blastomeres around the outside of the embryonal carcinoma cells, the embryonic cells, according to the inside-outside hypothesis (Hillman, Sherman, and Graham, 1972), would be encouraged to form

trophoblast while the embryonal carcinoma cells would be more likely to form the majority of the inner cell mass.

The design of these experiments is diagrammed in figure 1. After the embryonal carcinoma cells were aggregated with cleavage stage embryos, their contribution to the developing composite embryo was analyzed at three different stages by assaying for GPI isozymes. Initially, the aggregate embryos were cultured for 48 hours and the resulting blastocysts were analyzed. Subsequently, development *in utero* was examined by transferring aggregate embryos to pseudopregnant females and collecting the fetuses that had grown at 10-1/2 days gestation. Finally, aggregate embryos were allowed to develop to term and tissues of the resulting mice were assayed for embryonal carcinoma cell derivatives.

The embryonal carcinoma cells used in this study proved unable to support normal development without the aid of a substantial proportion of host inner cell mass cells. It was not possible, therefore, to generate animals composed entirely, or even largely, of embryonal carcinoma cells. Aggregation with cleavage stage embryos, however, despite certain technical disadvantages, proved to be an effective method for incorporating embryonal carcinoma cells into chimeric embryos and mice.

MATERIALS AND METHODS

Embryonal Carcinoma Cell Lines.

PSA-1, the embryonal carcinoma cell line used initially in this project was clonally derived from the tumor OTT 5568 (Martin and Evans, 1975). This is an experimentally induced tumor originating from a 3-day old, white-bellied agouti 129/Sv Sl^{j} C P embryo that had been grafted under the testis capsule of an adult mouse (Stevens, 1970). PSA-1 differentiates into a wide variety of tissues *in vivo* and forms cystic embryoid bodies which mimic early embryonic stages *in vitro* (Martin et al, 1977). It is trisomic for chromosome 6 and contains only one sex chromosome (XO)(Martin et al, 1978).

NG-2 was derived from PSA-1 by mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine followed by selection for HPRT-deficiency in 6-thioguanine (Dewey et al, 1977). Except for its HPRT deficiency, NG-2 resembles PSA-1 in all respects. It has the same karyotype and behaves similarly both *in vivo* and *in vitro*.

LT1-2D was clonally derived from the spontaneous ovarian tumor LT72484. This tumor had been transplanted *in vivo* 34 times before it was obtained from Dr. Leroy Stevens and the cell line was isolated. It has a modal number of 40 chromosomes with some shift to 41 when it differentiates. Its only visible chromosomal abnormality is an elongated chromosome 8 (Nobuo Takagi, personal communication).

All of the above cell lines were maintained on mitomycin-C treated feeder layers of mouse embryo fibroblasts (STO cell line from Alan Bernstein of the Ontario Cancer Institute) in Dulbecco's modified

Eagle's medium supplemented with 10% calf serum (selected batches). They were grown at 37°C in 5% CO_2 in air and were passaged every 3-4 days by plating 5 X 10^6 cells on 100 mm tissue culture dishes previously seeded with 5 X 10^6 feeder cells. For aggregation with embryos, the cells were harvested from confluent plates using 0.05% trypsin with EDTA and following standard cell culture procedures for passaging cells. To remove residual feeder cells which had survived the trypsinization procedure, 3 X 10^7 cells were plated on 100 mm tissue culture dishes for 30 minutes. During this time any residual feeder cells attached to the dish, whereas most of the embryonal carcinoma cells did not. These unattached embryonal carcinoma cells were collected and 10^6 cells were seeded on 35 mm bacteriological dishes. These were placed in the incubator overnight, during which time the cells formed clumps of various sizes. The smallest of these clumps were used for aggregations.

Mouse Embryos.

The inbred strain, SWR/J, was chosen for use in this study because cells of this strain are homozygous for Gpi-1b and thus distinguishable from the embryonal carcinoma cells used, which were all derived from strains homozygous for Gpi-1a. In addition, SWR/J mice are albino. This simplified identification of agouti coat coat color contributions by NG-2 in liveborn chimeric mice. SWR/J mice were obtained from Jackson Laboratories (Bar Harbor, Maine). In addition to SWR/J males, random bred ICR (swiss albino) males from Simonsen (Gilroy, California) were also used for some experiments as described in the results. These were analyzed for GPI type and only those homozygous for Gpi-1b were selected

for use.

To obtain embryos, females were superovulated by injecting 5IU PMS (Pregnant Mare's Serum, Sigma) intraperitoneally followed 48 hours later by 5IU HCG (Human Chorionic Gonadotropin, Sigma). Following the injection with HCG, female SWR/J mice were mated either with SWR/J males or with ICR Gpi-1b males and checked for vaginal plugs the next morning.

For the initial experiments in this project, two-cell stage embryos were collected by flushing the oviducts of pregnant females on the first day after the identification of a vaginal plug. The embryos were exposed to 0.5% pronase (Calbiochem, grade B) in warm phosphate buffered saline for 2-3 minutes to remove their zonae pellucida (Mintz, 1962). After exposure to pronase each embryo was individually transferred to a drop of Biggers standard egg culture medium (Biggers et al, 1971; Spindle and Pedersen, personal communication) under mineral oil (selected batches). There, the component blastomeres of each embryo were separated by passing the embryo through a fine bore, hand-pulled, micropipet. All of the blastomeres from a single embryo were cultured together in the same drop overnight at 37° in 5% CO_2 in air. Two groups of four blastomeres each, originating from the same embryo, were used to surround clumps of embryonal carcinoma cells.

For most of the experiments in this project, eight-cell stage embryos were flushed from the oviducts and uteri of pregnant females two days after identification of a vaginal plug. As described above, the embryos were exposed to 0.5% pronase in warm phosphate buffered saline for 2-3 minutes to remove their zonae pellucida. After exposure to pronase, they were rinsed six times for five minutes each in modified

Biggers' standard egg culture medium and placed in drops of the same medium under mineral oil until they were used in the aggregations.

Aggregations.

Aggregations were done in modified Hanks' BSS (Leibovitz, 1963; Spindle and Pedersen, personal communication) using phytohemagglutinin (PHA) (Bacto Phytohemagglutinin P, Difco). PHA is a mucoprotein from the red kidney bean, Phaseolus vulgaris, which has been used extensively in embryo-embryo aggregations. Its ability to agglutinate erythrocytes and leukocytes is blocked by N-acetyl-D-galactosamine (Borberg et al, 1966). PHA allows firm adhesion at room temperature and appears not to interfere with normal development (Mintz et al, 1973). It was used at a concentration of 0.2 micrograms per milliliter in warm Hanks' BSS. After association of the embryonal carcinoma cells with one or two embryos in warm PHA-containing medium, the aggregates were rinsed three times in modified Eagle's medium (Eagle, 1955; Spindle and Pedersen, 1973) containing 10% fetal calf serum (Hy Clone, Logan, Utah) and cultured overnight in drops of the same medium under mineral oil at 37°C in 5% CO_2 in air. It was found that the embryonal carcinoma cells do not survive well in medium without serum and that the embryos are more viable in utero if cultured in Eagle's medium rather than in Dulbecco's modified Eagle's medium.

In the early experiments, the aggregates were cultured for two days and the resulting blastocysts were collected for GPI analysis. In some cases, the blastocysts were subjected to immunosurgery (Solter and Knowles, 1975) so that isolated inner cell masses could be analyzed for

GPI phenotype. In subsequent experiments, the aggregates were cultured for one day to allow them to compact and form morulae at which time they were surgically transferred to the uteri of pseudopregnant females (foster mothers). They were then allowed to develop in utero for approximately eight days. Midgestation fetuses were dissected from the uteri of pregnant foster mothers, rinsed in phosphate buffered saline, photographed, and analyzed for GPI phenotype. In some cases, the aggregates were allowed to develop to term in utero. In these cases fifteeen tissue samples each were collected for GPI analysis from liveborn mice exhibiting no coat color mosaicism. See figure 1 for a diagram of the above experiments.

Transfer to Pseudopregnant Females.

Random bred CD-1 mice from Charles River were used for foster mothers. Before use, these mice were innoculated with Sendai vaccine (Microbiological Associates, Los Angeles) and were quarantined for two weeks. One day after the SWR/J females were mated, CD-1 females in estrus were mated with vasectomized males. This one day time lag compensated for the slower development of the aggregate embryos due to in vitro culture and, perhaps, to the number of manipulations involved in the aggregation process. Compacted aggregates were surgically transferred to foster mothers two days after the detection of a vaginal plug. During this procedure the females were anesthetized with Avertin, a mixture of tribromoethanol and tertamyl alcohol (Gearhart, personal communication). This anesthetic was found to be very effective, acting quickly and resulting in no visible aftereffects or complications.

Glucose Phosphate Isomerase Assay.

As mentioned earlier, all of the embryonal carcinoma cells used in this study were derived from strains homozygous for Gpi-1a. The embryos used, SWR/J, were all homozygous for Gpi-1b. The isozymes encoded by these two glucose phosphate isomerase (GPI) alleles were identified by electrophoretic separation and visualization with a tetrazolium salt-linked activity stain according to the procedure of Eppig et al (1977). Electrophoresis was done in tris-glycine buffer, pH 8.6, at 250 volts for one hour. Under these conditions GPI migrates toward the cathode, the Gpi-1a isozyme migrating more slowly than the Gpi-1b isozyme. For smaller samples, such as blastocysts and inner cell masses, Zip Zone cellulose acetate plates (Helena Laboratories, Beaumont, Texas) were used as a matrix. The samples were lysed in small drops of 1% triton X in buffer and loaded with small bore, glass micropipets. For assays of larger samples, such as tissues, sheets of Cellogel 200 (Chemtron, c/o Kalex Scientific Company, Inc., Manhasset, New York) were used and homogenized samples were applied with a Gelman Sepratek-8 applicator (Gelman Instrument Company, Ann Arbor, Michigan).

For midgestation samples, when two bands were present, their intensity relative to each other was estimated by eye (greater than, equal to, or less than) as illustrated in figure 9. This was to make possible the classification of the samples into categories which would crudely reflect the extent of embryonal carcinoma cell contribution.

Photomicrographs.

Phase contrast photomicrographs of the aggregates and embryos were

taken with an inverted Wild M40 phase microscope. Photomicrographs of freshly dissected fetuses were taken with a Wild stereomicroscope equipped with a Nikon camera. Photomicrographs of two micron sections were taken with a Zeiss microscope. Kodak Royal X Pan film was used for the phase contrast photomicrographs. Kodak Plus X Pan film was used for all of the other photomicrographs.

Thick Sections.

Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer for 60 minutes after which they were rinsed three times in 0.1 M sodium phosphate buffer, dehydrated in ethanol, and embedded in glycol-methacrylate (DuPont Sorvall). Polymerized blocks were sectioned on a JB4 microtome (DuPont Sorvall). Two micron sections were retained every ten to twenty microns throughout the length of the samples. These sections were mounted on a slide, stained with 1% thionine, coverslipped, and examined carefully for groups of displaced cells that might be identified as unintegrated embryonal carcinoma cells.

RESULTS

Figure 1 diagrams the experimental procedures described below. The aggregates were examined at three stages: as blastocysts, as midgestation fetuses, and as live animals.

Incorporation of Embryonal Carcinoma Cells into Blastocysts

The first experiments focused on maximizing the relative number of embryonal carcinoma cells in the inner cell masses of blastocysts. This was accomplished by separating SWR/J embryos at the two-cell stage into their component blastomeres, allowing the blastomeres to undergo two divisions, and then arranging the resulting two groups of four blastomeres each around a small clump of PSA-1 embryonal carcinoma cells in the presence of the lectin, phytohemagglutinin (PHA). These aggregates compacted into apparently normal morulae after 24 hours in culture. Blastulation occurred within 48 hours of aggregation (figure 2). Of 422 aggregates, 300 blastulated normally. This represents an overall yield of 71%, with a range from 36 to 93% per experiment.

The ability of the embryonal carcinoma cells to interact with embryonic cells to form aggregate blastocysts was most likely a consequence of the close similarity between the two cell types. However it is possible that aggregation and subsequent blastulation were general, non-specific phenomena attributable to the potent agglutinating ability of PHA. To test this, aggregates were made using differentiated mouse cells, STO or PYS, in place of the undifferentiated embryonal carcinoma cells. STO is an HPRT-deficient mouse fibroblast cell line (Martin and

Evans, 1975). PYS is a differentiated teratocarcinoma cell line which resembles parietal yolk sac endoderm (Lehman et al, 1974). Although initially these cells adhered to the embryo cells, within a few hours after removal from the PHA the aggregates fell apart (figure 3, B and E). Both the STO cells and the PYS cells subsequently attached to the plate (figure 3, C and F). Thus, although PHA serves to hold cells together initially, it does not arbitrarily ensure subsequent development of chimeric blastocysts.

To verify the continued presence of the embryonal carcinoma cells, blastocysts were assayed for embryonic (Gpi-1b) and embryonal carcinoma cell (Gpi-1a) glucose phosphate isomerase (GPI) activity. In most cases both forms of the enzyme were present (figure 4). When inner cell masses were isolated from such blastocysts and assayed only one band, corresponding to the PSA-1 Gpi-1a isozyme, was detectable in two out of three experiments (figure 4, e). Two bands were detectable in the third experiment. These results provide biochemical evidence of the presence of PSA-1 embryonal carcinoma cells in the experimental blastocysts.

Development of Embryonal Carcinoma Cell - Embryo Aggregates In Utero

Abnormal forms of development. In the above experiments, splitting the embryos and arranging their blastomeres around the outside of embryonal carcinoma cell clumps resulted in aggregate blastocysts with inner cell masses composed entirely of embryonal carcinoma cells in two out of three experiments. This achieved our original goal which was to maximize the contribution of the embryonal carcinoma cells to the inner cell masses of aggregate blastocysts. Unfortunately, these aggregate

blastocysts implanted at a very low frequency when transferred to the uteri of pseudopregnant females, presumably because the requirements for viability in utero are more stringent than those for development in vitro. This forced us to stop splitting the embryos both to decrease the amount of stress to which they were subjected and to shorten the necessary culture period. Since one of the objectives of this project was to improve incorporation by mutant embryonal carcinoma cells, the following experiments were done using NG-2, an HPRT deficient derivative of PSA-1.

Small clumps of embryonal carcinoma cells, corresponding in size to one eight-cell stage blastomere and containing approximately ten to twenty embryonal carcinoma cells, were aggregated each with one eight-cell stage embryo in the presence of PHA (figure 5). After culture overnight, they were transferred to the uterus of a pseudopregnant female. Eight days, or in a few cases seven days, later (approximately 10-1/2 days gestation), the females were sacrificed and any developing fetuses were removed for examination and GPI analysis. Once culture and manipulation techniques were refined to an extent that allowed successful implantation of the aggregates, it was found that the aggregates were not developing normally. In subsequent experiments several groups of abnormal phenotypes were observed. These ranged from only slightly deformed or retarded to grossly abnormal (figure 6). Roughly six groups were distinguished:

Group 6. In this category were grouped fetuses which were very nearly normal. Usually their only defect was a misshapen, sometimes stunted head. Otherwise, they often appeared to be normally formed 10-

1/2 day fetuses: They had well formed limb buds, apparently normal hearts, and clearly defined somites. In a few cases, this group may have included samples that were damaged during dissection.

Group 5. Fetuses in this group were clearly retarded although they often appeared normal in other respects. They had the appearance of 9-1/2 day fetuses including the typical kyphosis curve of later developmental stages, clearly developed mandibular arches, somites, apparently normal hearts, and the beginning of small limb buds. Occasionally a lump protruded from their caudal end. When embryonal carcinoma cell derivatives were shown to be present by GPI analysis, these were found in both the fetus proper and the lumps. The lumps, therefore, were not embryonal carcinoma cells that had segregated from the rest of the developing fetus but probably represented remnants of the allantois.

Group 4. This group was characterized by greater retardation than group 5 in addition to a greater degree of abnormality, as indicated by the wider variation in the phenotypes observed. The fetuses in this group were clearly less well developed than those of group 5, crudely resembling 8-1/2 to 9 day fetuses. They had not yet acquired a kyphosis curve and their neural tubes were not completely closed. The midgut appeared more or less closed and the heart had visibly begun to develop, protruding from the ventral side.

Group 3. Fetuses belonging to group 3 were distinguished from those in group 4 primarily by the degree of body closure. While the midguts of group 4 fetuses were nearly closed, the body walls of group 3 fetuses were contiguous with the yolk sac along most of their length. These fetuses resembled 8 day fetuses. When enclosed in the amnion, they

exhibited extreme lordosis. After dissection from the amnion, well developed somites were visible, arranged on either side of an open neural groove. Despite the obvious abnormal retardation of these fetuses and the abnormal configurations of their neural folds, for example, cross sections of a fixed and embedded group 3 fetus revealed structures similar to those of a normal 8 to 8-1/2 day fetus. No grossly displaced groups of cells were seen and the organization of the fetus appeared normal (figure 7).

Group 2. Group 2 fetuses were the most retarded and primitive of the structures that could be recognized as "fetuses." They were small and flat and roughly resembled development at 7-1/2 days. Enclosed in the amnion, a head process with crude cephalic structures was usually visible on one end while an allantoic stalk projected from the other. Again, sections of a fixed and embedded group 2 fetus revealed no cells that could be identified as unintegrated embryonal carcinoma cells (figure 8).

Group 1. This group was most typically portrayed by a lump on the yolk sac. In a few cases, development proceeded further, however it did so in a disorganized manner which resulted in a bizarre assortment of structures grouped together on the yolk sac. In figure 6, group 1, for example, a beating heart tube, deformed brain vesicles, and an allantoic stalk are shown haphazardly associated together. This group represents the most abnormal of the structures to develop from implanted aggregates.

The above groups were used in the following experiments to classify the degree of normal or abnormal development in utero. These observa-

tions were then compared with the degree of chimerism in each sample as roughly indicated by the GPI assays (see methods and figure 9).

Development of embryo controls. When it became apparent that the aggregates were developing abnormally in utero, the first, and most obvious, question was whether this abnormal development was caused by the aggregation technique or whether it represented a deficiency in the developmental capabilities of the embryonal carcinoma cells. Accordingly, control experiments without embryonal carcinoma cells were conducted, the results of which are represented in figure 10 and tabulated in tables 1 and 2. First, mock experiments were done in which SWR/J embryos were processed through the entire aggregation procedure to test the effects on development of the culture process, pronase, culture media, PHA, and experimental manipulations. Of these, only 2 out of 34 (6%) showed any developmental defect at 10-1/2 days of gestation, and these fetuses displayed the more advanced, only slightly retarded, phenotypes.

Next, aggregations were done with a variety of embryonic cell types to test the role of embryonic stage difference in disturbing the development of the aggregates. Since NG-2 was derived from a tumor that arose from a 129/SvSl embryo, embryos from the most closely related strain readily obtainable, 129, were used for these experiments. In the first experiments, eight-cell embryos of the strain 129 were aggregated each with one SWR/J eight-cell embryo. In all cases, development was completely normal.

In the next set of experiments, inner cell masses isolated from 129

blastocysts were aggregated with SWR/J eight-cell embryos. Most of these developed normally, although a few, 5 out of 21 (24%), were abnormal at 10-1/2 days. Interestingly, many of the developing fetuses were composed entirely of either SWR/J cells or 129 cells. Only 2 out of 21 (10%) contained cells from both strains.

Since embryonal carcinoma cells are thought to be most analogous to embryonic ectoderm cells, the most appropriate control experiment appeared to be aggregation of embryonic ectoderm cells with eight-cell embryos. Inner cell mass cells which had been cultured for two days and subjected to a second immunosurgery to remove their outer layer of endoderm were used in place of embryonic ectoderm cells dissected from early post-implantation embryos. Cells obtained in this manner are thought to be analogous to embryonic ectoderm cells and are much easier to obtain in quantity. In addition to their close resemblance to embryonal carcinoma cells both in their morphology and in their behavior in vitro, one-dimensional polyacrylamide gels, comparing the protein patterns of such cultured inner cell masses to those of embryonal carcinoma cells, show them to be remarkably similar (Martin et al, 1978). Cultured and immunosurgerized 129 inner cell masses were aggregated with SWR/J eight-cell embryos and the compacted aggregates were transferred to the uterus of a pseudopregnant mouse. Because these cultured 129 ICM cells were difficult to obtain, in part due to the lengthy culture period and additional manipulations involved, and in addition because these aggregates did not compact well in culture, implantations from only a small number of aggregates were examined. Compared with the other control embryonic cell types, embryonic ectoderm

contributed to many fewer midgestation fetuses. Only 5 out of 17 (29%) contained 129 cells at 10-1/2 days. Furthermore, many more implantations, 10 out of 17 (59%), were found to be abnormal. This abnormal development occurred regardless of whether the fetuses contained 129 embryonic ectoderm cell derivatives. Normal development by the 129 embryonic ectoderm cells was not precluded since one of the normal fetuses was found to be composed entirely of 129 cells.

Development of NG-2 - embryo aggregates. Although it was found that some control aggregates with normal embryonic cells displayed abnormal development, the degree of abnormality expressed by the NG-2 aggregates was much more severe (table 1). Furthermore, those aggregates that did develop abnormally contained extensive embryonal carcinoma cell contributions, in most cases equaling or exceeding that of the SWR/J embryonic cells. This, taken together with the fact that one normally developed aggregation chimera had been observed, suggested that more normal chimeras might be obtained by decreasing the level of NG-2 contribution to each aggregate. The results of these experiments are summarized in table 1 and figure 11.

The first approach was to use fewer NG-2 cells in the aggregations. Smaller NG-2 clumps were used, containing 5 to 10 cells rather than 10 to 20 cells (figure 12, B). Using smaller NG-2 clumps did not appreciably improve development, so aggregates were made using doublets or triplets of NG-2 cells. NG-2 participation in the resulting aggregates was decreased to such a great extent that the incidence of chimerism fell from 60% to 8%. Since this low incidence of chimerism was unac-

ceptable, a different approach was taken: The number of embryonic cells in the aggregates was increased.

NG-2 clumps of the original size (10 to 20 cells) were aggregated with two SWR/J eight-cell embryos (figure 13). Contribution by the NG-2 cells decreased slightly and, concomitantly, development improved somewhat. At this time, the poor mating performance of the SWR/J males caused us to begin using random bred ICR males which had been shown to be Gpi-1b. Embryos containing a random bred component in their background, i.e. ICR X SWR/J, contributed much more extensively than inbred embryos to NG-2 - embryo aggregates. The consequence of this was that the extent of embryonal carcinoma cell contribution was decreased (table 2, #B≷A/#+A). This shift was accompanied by a substantial improvement in development (table 1). Of 13 chimeras that developed from the aggregation of a clump of NG-2 cells with two ICR X SWR/J eight-cell embryos [NG-2 - 2(ICRXSWR) aggregates], 4 (31%) were completely normal. Attempts to develop this trend further by using three instead of two embryos did not visibly improve development and thus did not justify the increased incidence of intersexes and the decreased viability associated with the use of three embryos. In experiments in which clumps of NG-2 cells were aggregated with one ICR X SWR embryo, the incidence of NG-2 incorporation was low, 2 out of 14 (14%). Although development was somewhat improved, it was still abnormal (table 1). The optimum combination appeared, therefore, to be one clump of NG-2 cells with two ICR X SWR embryos.

The results of this series of experiments, as summarized in figure 11, suggest that as the contribution of the embryonal carcinoma cells to

each aggregate decreases, normalcy of development increases. Using the data from figure 11, a statistical estimation of the strength of this relationship and its significance can be obtained. Cramer's statistic is a modification of the contingency coefficient which was used to measure the strength of the inverse correlation between NG-2 contribution and normalcy of development (Roscoe, 1969). For the data in figure 11, the Cramer's statistic is .78 (the closer to 1, the stronger the correlation). This signifies a reasonably high degree of relationship. According to the chi-square test of independence, the probability that this relationship is due to chance is less than .005. In contrast, a similar chi-square test of the embryo control data (grouped) indicates a probability of randomness of .75.

Apparently NG-2 cells require a certain amount of assistance from embryonic inner cell mass cells to form a normal fetus. To learn whether this is also true for other embryonal carcinoma cells, aggregations were begun with two other cell lines, PSA-1 and LT1-2D.

Development of aggregates containing PSA-1 or LT1-2D cells. PSA-1 was chosen because it is the cell line from which NG-2 was derived. Although it has the same karyotype as NG-2, trisomy 6 and XO, it has not been subjected to either mutagenesis or selection. LT1-2D was chosen because it has an almost normal karyotype, its only detectable abnormality being an elongated chromosome 8 (Takagi, personal communication). Since the purpose of these experiments was to determine whether aggregates composed largely of PSA-1 or LT1-2D derivatives would develop abnormally, aggregations were done with one SWR/J eight-cell embryo. It

was in this combination that NG-2 contributed most extensively to aggregates and concomitantly participated most often in abnormal development.

The results of these experiments are summarized in figure 14 and indicate that in aggregations with both PSA-1 and LT1-2D, as well as NG-2, normal development is correlated with embryonal carcinoma cell contribution to less than half of the fetus. The Cramer's statistic for these data is .68, not quite as high a degree of relationship as expressed by the NG-2 data, but nevertheless still significant. Again, the chi-square test of independence indicates less than .005 probability of randomness (Roscoe, 1969).

Neither PSA-1 nor LT1-2D survive as well as NG-2 in aggregates developing in utero (table 2A, #+A/#tot): 58% of the aggregations with PSA-1 still contain PSA-1 cells after eight days in utero and only 40% of the aggregations with LT1-2D still contain LT1-2D cells. This is compared to an NG-2 frequency of contribution at midgestation of 79%. In addition, PSA-1 and LT1-2D appear to contribute less extensively to each aggregate (table 2B, #B≷A/#tot+A). In the aggregates that still contain embryonal carcinoma cells after eight days in utero, the embryonal carcinoma GPI isozyme was present in greater amounts than the normal embryo SWR/J isozyme in 53% of the aggregates made with PSA-1 and in 75% of the aggregates made with LT1-2D. This is compared with 93% of the aggregates made with NG-2 in which this occurred.

With respect to development, PSA-1 appears to be better able to participate in normal development than NG-2 or LT1-2D. 42% of the aggregates still containing PSA-1 cells after eight days in utero were normal

fetuses compared to 17% of the LT1-2D aggregates and 7% of the NG-2 aggregates (table 3, #norm+A/#tot+A). This is, in part, a reflection of the lesser contribution by PSA-1 cells to each aggregate. As mentioned previously, PSA-1 contributes less extensively than LT1-2D, which in turn contributes less extensively than NG-2.

If one compares only those aggregates in which the embryonal carcinoma cells contributed less than half of the fetus (table 3, #normB>A/#totB>A), one still finds that PSA-1 chimeras develop normally most often. The small number of samples in this category makes the strictly analogous comparison with NG-2 aggregates containing only one SWR/J embryo misleading. Embryonal carcinoma cells contributed less than half of the fetus in only one of these chimeras. Comparison of PSA-1 aggregates with NG-2 - 2(ICRXSWR) aggregates, in which the embryonal carcinoma cells participated less extensively, however, confirms that more PSA-1 chimeras are normal. Similarly, LT1-2D chimeras also develop normally more often than do NG-2 - 2(ICRXSWR) chimeras. Thus there appears to be some difference in developmental capabilities between the three cell lines which is unrelated to their ability to survive and dominate in a chimeric situation.

Development of Embryonal Carcinoma Cell - Embryo Aggregates to Birth

Since a reasonable number of normal, midgestation chimeras developed from NG-2 - 2(ICRXSWR) aggregates, further experiments were conducted in which the aggregates were allowed to develop to term. Mice born of experimental foster mothers (pseudo-pregnant females who had received experimental embryos by surgical transfer, see methods) were

allowed to grow until their coats were fully developed. Those not exhibiting coat chimerism, i.e. containing no obvious agouti hairs in their otherwise albino coats, were sacrificed and 15 tissue samples from each were analyzed for GPI chimerism.

A total of 25 animals were born. This represents a frequency of development to birth of 13% (25/190). This frequency of development to birth of NG-2 - 2(ICRXSWR) aggregates is comparable to their frequency of normal development to midgestation (15%) (table 4, #normal/#trans). Their frequency of normal development to midgestation is less than that of mock processed (27%), 129 morulae (37%), and 129 ICM (22%) aggregates. It is, however, greater than that of 129 embryonic ectoderm aggregates (7%). This is a reflection of the lower implantation frequency of 129 embryonic ectoderm aggregates. The implantation frequency of these aggregates is half that of the NG-2 - 2(ICRXSWR) aggregates. When one considers only those aggregates which have implanted, the two combinations exhibit similar frequencies of normal development (table 3, #norm/#tot).

Of the 25 animals that were born from NG-2 - 2(ICRXSWR) aggregates, one died and could not be retrieved for GPI analysis. Among the remaining 24, 16 were male and 8 were female. One, a male, had a large patch of agouti hairs on the right side of his face and at least one stripe on his back (figure 16). Seven of the twenty-four mice contained embryonal carcinoma cell contributions to one or more of their tissues, as ascertained by GPI analysis (figure 15, table 5). This is a frequency of incorporation of 29% which is lower than that found in normal fetuses at midgestation (44%) (table 4, #norm+A/#norm tot).Comparing only mid-

gestational results, the rate of incorporation into normal fetuses of NG-2 - 2(ICRXSWR) aggregates (44%) is comparable to that of PSA-1 - SWR aggregates (40%) and higher than that of LT1-2D - SWR aggregates (20%) and NG-2 - SWR aggregates (20%).

GPI analysis showed the presence of embryonal carcinoma cell derivatives in several tissues (table 5). The number of chimeric tissues in each mouse varied from one to eight. Embryonal carcinoma cells contributed most often to the heart and gut (stomach) and contributed most extensively to the heart, liver, and pancreas. Only one of the 24 animals had a tumor. As evaluated by GPI analysis, this tumor contained both embryo and embryonal carcinoma derivatives in roughly equal amounts.

DISCUSSION

Incorporation of Embryonal Carcinoma Cells into Blastocysts

This study was designed to determine to what extent embryonal carcinoma cells would form a normal mouse and, if possible, to increase the overall contribution of embryonal carcinoma cells to chimeric mice. This was to be done by aggregating the cells with cleavage-stage embryos. The first part of the study established the critical point that this approach was, in fact, feasible. Not only did embryonal carcinoma cells aggregate readily with cleavage-stage embryos but the aggregates consistently formed apparently normal blastocysts. Furthermore, when surrounded by eight-cell stage blastomeres, the embryonal carcinoma cells often formed the major portion of, and sometimes the total, inner cell mass. Even in later experiments, when aggregated with two intact eight-cell stage embryos, the embryonal carcinoma cells often formed a substantial proportion of the ICM in the cases when these were analyzed. These results demonstrate that aggregation with cleavage-stage embryos is a simple and effective method for obtaining quite extensive incorporation of embryonal carcinoma cells into early mouse embryos.

These experiments used biochemical glucose phosphate isomerase isozyme markers to identify the embryonal carcinoma cell derivatives. A morphological study in which ^{3}H-thymidine was used to identify the host embryo cells reported similar results: F-9 embryonal carcinoma cells were found to aggregate readily with eight-cell stage embryos whereas PYS and STO cells did not. Embryonal carcinoma cells were identified in sections of aggregate blastocysts and usually appeared integrated into

the inner cell mass (Stewart, 1980).

Development of Embryonal Carcinoma Cell - Embryo Aggregates In Utero

Abnormal development and embryo controls. Once it was found that embryonal carcinoma cells would incorporate into blastocysts after aggregation with cleavage-stage embryos, the next step was to determine whether these aggregate blastocysts could continue to develop in utero. The results of early experiments showed that they could, indeed, implant and develop when transferred to the uterus of a pseudopregnant female. Development, however, was often abnormal. Control experiments with mock processed embryos indicated that these abnormalities were not caused by the culture conditions, culture media, pronase, PHA, or the experimental manipulations per se. With the exception of the experiments involving 129 embryonic ectoderm, the implantation rates of both experimental and control aggregates resembled those of the mock processed embryos, varying around 30% (table 4). Aggregation with embryonal carcinoma cells did not seem to adversely affect implantation.

Not surprisingly, control aggregations between strain 129 eight-cell embryos and SWR eight-cell embryos developed normally. Interestingly, the results of these aggregations suggested a possible strain imbalance since SWR cells predominated in the chimeras and 3 out of 7 fetuses contained only SWR cells. The sample size was, however, too small to draw any firm conclusions on this point.

In contrast to the control experiments with cleavage-stage embryos, in aggregations of 129 ICMs with SWR eight-cell embryos, the 129 cells predominated (figure 10). 62% (13/21) of the developing aggregates

contained only 129 cells at 10-1/2 days of development, while most of the rest contained only SWR cells. Only 10% (2/21) contained cells from both strains. The reason for this imbalance is unclear. The experiments with cleavage-stage embryos show that a strain imbalance favoring 129 cells was not the cause. It is possible that the 129 ICM cells had an advantage due to their more advanced stage of development. Contribution by the 129 ICM cells to the inner cell masses of developing aggregate blastocysts may have been favored because the ICM cells were already at the appropriate stage. In seeming conflict with this suggestion are the results obtained by Rossant (1975) when she aggregated ICM cells from an inbred strain with morulae from random bred CFLP. Most of the fetuses that she analysed at midgestation contained cells of both strains and neither strain predominated. However, since the morulae cells in this case were random bred, their greater hybrid vigor may have counteracted the possible developmental stage advantage of the ICM cells. In contrast, inbred SWR cleavage-stage embryos, although also possibly possessing a strain advantage, were not able to compete with 129 ICM cells.

Considering the vigorous contribution of 129 ICMs to aggregate blastocysts, together with the earlier results indicating extensive contribution by embryonal carcinoma cells to aggregate blastocysts, one might expect that 129 embryonic ectoderm cells would also incorporate easily and contribute widely to subsequent tissues. This appears not to have been the case. Embryonic ectoderm cells survived and developed much more poorly than other normal 129 embryonic cell types when aggregated with SWR eight-cell embryos, contributing cells to only 5 out of 17 fetuses (29%) at 10-1/2 days (table 2, #+A/#tot). The embryonic ectoderm

cells used in these experiments were subjected to a much longer period of *in vitro* culture than the other embryonic cell types. In addition they underwent immunosurgery twice and in a few instances were broken into smaller groups of cells by passage through a small bore glass micropipet. These additional hardships may have significantly decreased their viability relative to the other embryonic cells used in previous control experiments. This may explain their decreased survival in developing aggregates. When the 129 embryonic ectoderm cells did survive, they appeared to compete well with the SWR cells. In 3 out of 5 cases they composed the entire fetus. This is consistent with the observation from 129 ICM experiments that more advanced embryonic cells are at an advantage in these aggregation chimeras.

The frequency of implantation was low and development in general was poor in the experiments involving the combination of 129 embryonic ectoderm with SWR embryos. Only 17 out of 101 aggregates (17%) that were transferred implanted and developed (table 4). This is considerably lower than the implantation frequency in any of the other experiments in this study and may reflect an unrecognized problem in the culture of these aggregates. Or, the damaged embryonic ectoderm cells, being unable to incorporate effectively, may have perturbed the aggregates enough to prevent successful implantation. As mentioned in the results, many of these combinations did not compact well. Although uncompacted aggregates were discarded, it is possible that in many of the aggregates which were transferred, the two groups of cells were not well integrated despite their compact appearance. This might then have been reflected in the subsequent inability of the aggregates to implant. Whatever the reason

for this low frequency of implantation, it seemed not to be a factor in other experiments since frequencies of implantation were relatively uniform throughout the rest of the study.

Of the seventeen 129 embryonic ectoderm - SWR embryo aggregates that did implant, only seven (41%) were normal. This is a relatively low level of normal development and may be a sign that combinations between embryo cells which differ too greatly in developmental stage will not do well. This is also suggested by the results of the 129 ICM experiments in which only 16 out of 21 (76%) developed normally. As the difference in developmental stage between the members of an aggregate increased, normal development decreased (table 3, #norm/#tot). Disturbance in development appears not to involve the continued presence of both cells in the combination however, since the occurrence of abnormal development was not correlated with the survival of both cells in the aggregate. In fact, in all of the cases of abnormal development involving 129 ICM cells and in 8 out of 10 of the cases involving 129 embryonic ectoderm cells, only one of the strains was present (table 1). Neither strain was more or less often associated with abnormal development than the other strain.

It appears from the above results that the considerable difference in developmental stage between embryonal carcinoma cells and eight-cell stage embryos might at least partially account for the abnormal development found in the early experiments with NG-2 (NG-2 plus one SWR eight-cell stage embryo). In spite of this, several points suggest that the developmental potential of the embryonal carcinoma cells themselves was also involved. In the above control experiments, abnormal devel-

opment was not correlated with the level of contribution by any one embryonic cell type or strain to a particular aggregate. On the contrary, for each of the embryonic cell types, including 129 embryonic ectoderm, normal 10-1/2 day fetuses were recovered which were composed entirely of derivatives of that cell type, demonstrating the ability of the cells to direct normal development. In contrast, abnormal development by NG-2 - SWR aggregates was clearly associated with large contributions by the NG-2 cells. Most of the aggregates containing no NG-2 cells developed normally (table 3, #norm allB/#tot allB). Those containing large numbers of NG-2 cells always developed abnormally (table 3, #norm B≷A/#tot B≷A). Moreover, the incidence of abnormal development (74%) was higher than that of 129 embryonic ectoderm aggregates (59%), and the phenotypes were uniformally more severely abnormal than those found in controls (table 1).

Development of NG-2 - embryo aggregates. As described in the results, attempts to decrease the proportion of NG-2 cells in the aggregates by decreasing the number of cells used in the original combination were not successful. The use of smaller groups of cells did not appreciably decrease NG-2 contribution and the use of doublets or triplets eliminated NG-2 contribution. Embryonal carcinoma cells in general do not survive well in isolation. This may explain why aggregations with doublets and triplets failed.

Attempts to decrease the level of NG-2 participation by increasing the number of embryos used were more successful. The use of two SWR embryos significantly reduced the proportion of NG-2 cells in the

developing aggregates. The observation that development was also shifted, toward more normal development, confirmed the prediction that development would improve when NG-2 participation was decreased. This was further, and more clearly, demonstrated by the results from the experiments in which ICR X SWR embryos were used. In these experiments, the embryo cells were able to compete very well and in the majority of the cases formed the major portion of the developing fetus (figure 11). At the same time, development was dramatically improved: The incidence of normal development among those aggregates that contained NG-2 cell derivatives increased from 7% to 31% (table 3, #norm+A/#tot+A).

The experiments with ICR X SWR embryos suggested that some facet of "hybrid vigor" enabled the embryo cells to compete more successfully with the NG-2 cells in forming the aggregate embryo. That the genotype of an embryo will influence its relative contribution to a composite embryo has been observed previously in aggregations between normal embryos. Mullen and Whitten (1971), in studies involving aggregations between inbred strains, report that in some strain combinations one strain will dominate. The results of the present study suggest a possible strain imbalance in the experiments involving the aggregation of SWR with 129 cleavage-stage embryos. Thus, it is not surprising that adding an element of outbreeding to the genetic background of the embryos increased their contributions in aggregations with NG-2.

In addition to allowing the embryos to compete more successfully, the ICR strain component in their genetic background may have enhanced the effectiveness of their association with the NG-2 cells. This was examined briefly in experiments in which NG-2 cells were aggregated with

one ICR X SWR eight cell embryo. Curiously, the frequency of incorporation of NG-2 cells into aggregates with one ICR X SWR embryo was low (14%) compared to incorporation into aggregates with two ICR X SWR embryos (62%). One possible explanation is that the NG-2 cells were more often excluded from association with one ICR X SWR embryo than from association with two embryos because they are more easily trapped between two embryos. In the two cases in which NG-2 cells did survive in association with one ICR X SWR embryo, development was less abnormal than in associations with one SWR embryo (table 1). However, the extent of their incorporation was also decreased in these combinations (table 2, #B$\geq$A/#tot+A). Thus, while it is possible that the ICR X SWR embryos were better able to assert a normalizing influence on the NG-2 cells, this ability was not clearly distinguishable from their ability to compete more successfully and thereby prevent the NG-2 cells from dominating the aggregates.

This series of experiments established that a substantial number of the composite embryos resulting from aggregations between NG-2 cells and cleavage-stage embryos would develop normally given the optimal sort of combination. This was found to be the combination of a clump of 10-20 NG-2 cells with two ICR X SWR embryos. Combinations involving one or three embryos were not as successful. As described earlier, development of aggregates containing one ICR X SWR embryo continued to be abnormal, although somewhat less severely so. Development of aggregates containing three ICR X SWR embryos, although as normal as that of aggregates containing two embryos, did not show enough improvement to justify the greater risk of perturbations resulting from intersex combinations.

The data suggest, as illustrated in figure 11, that the reason that the combination involving two ICR X SWR embryos develops more normally than others is that the level of participation by the NG-2 cells in these aggregates is decreased to an extent which allows normal development without precluding the continued survival of the NG-2 cells. The inference is that NG-2 cells are somehow deficient in their ability to direct normal development and require the assistance of a substantial number of normal embryonic cells. If this is true, it raises questions regarding why the NG-2 cells are developmentally deficient and whether all embryonal carcinoma cells are similarly deficient.

Development of aggregates containing PSA-1 or LT1-2D cells.

Experiments were begun with PSA-1 and LT1-2D in an attempt to partially answer these questions. Aggregations were done with one SWR eight-cell embryo, since it was in this combination that the abnormal development associated with large proportions of NG-2 cells was most apparent. The results of these experiments are summarized and analyzed in tables 1 through 3 and in figure 14.

Since NG-2 had been subjected to mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine and selection in 6-thioguanine, it was thought that experiments with its parent cell line, PSA-1, might indicate whether or not these procedures had had any detrimental effect on the developmental capabilities of NG-2. Interestingly, PSA-1 did not contribute either as often or as extensively to developing aggregates as did NG-2 in analogous situations (table 2). The decreased participation by PSA-1 in aggregates with SWR embryos resulted in much more normal development by

the aggregates. Decreased participation was not the only reason for better development by the PSA-1 aggregates however. If PSA-1 - SWR aggregates are compared with NG-2 - 2(ICRXSWR) aggregates in which NG-2 contribution was much less extensive, lower even than that of PSA-1, it is found that the PSA-1 aggregates still developed normally more often than NG-2 aggregates (table 3, #norm+A/#tot+A). In comparisons of only those samples in which embryonal carcinoma cells contributed less than half of the fetus, PSA-1 aggregates again appear to develop normally more often (table 3, #norm B>A/#tot B>A). From this, it appears that NG-2 is less able to direct normal development in association with normal embryo cells than is PSA-1. This suggests that mutagenesis and selection can have detrimental effects on developmental capabilities, both by directly decreasing the cell's ability to allow normal development and by increasing its ability to grow in competition with other cells in a composite embryo.

Although more PSA-1 aggregates developed normally than did NG-2 aggregates, many still developed abnormally. Since both PSA-1 and NG-2 are trisomic for chromosome 6 and are XO, it is possible that their abnormal karyotype was responsible for the abnormal development of the aggregates. LT1-2D is a cell line whose only detectable karyotypic abnormality is an elongated chromosome 8. These cells were aggregated with SWR embryos to determine whether their more normal karyotype would allow normal development more often. This proved not to be the case. Although LT1-2D cells participated in normal development more often than did NG-2 cells, they did not do so more often than PSA-1 cells (table 3, #norm+A/#tot+A). In addition, development in aggregates no longer

containing embryonal carcinoma cells was normal less often than in previous experiments with NG-2 and PSA-1 (table 3, #norm allB/#tot allB). This trend in the LT1-2D experiments of abnormal development by aggregates containing only SWR cells is similar to that observed in the 129 embryonic ectoderm experiments. Whether a similar mechanism is involved is unclear. However, it appears that the presence of LT1-2D cells in an aggregate, like that of 129 embryonic ectoderm cells, disturbs subsequent development even when the cells are no longer present.

The above results with LT1-2D established three points: The first is that the trisomy 6 in NG-2 and PSA-1 cells, although possibly a contributing factor, is most likely not the principle cause of the abnormal development of aggregates containing these cells. Not only were the abnormal phenotypes very different from those seen in trisomy 6 fetuses (see Gropp, 1975), but aggregates containing LT1-2D cells, cells not trisomic for chromosome 6, showed similar patterns of abnormal development.

The second point is that a nearly normal karyotype is not sufficient for normal development. Although LT1-2D cells were capable of normal development in conjunction with normal embryonic cells, as in experiments with NG-2 and PSA-1, development was abnormal when the contribution by embryonal carcinoma cells was large. This is in agreement with the studies of other investigators (Papaioannou et al, 1979) who found that a normal karyotype will not necessarily ensure widespread incorporation of embryonal carcinoma cells into normal mice. Whether the other differences that were observed between the behaviour of LT1-2D and

PSA-1 in aggregates (lowered incidence but increased extent of contribution by LT1-2D cells) were caused by the differences in their karyotype or by differences in other characteristics cannot be determined from these experiments.

The third point is that the tendency to disturb development when present in large proportions in aggregates is a characteristic that is shared by two embryonal carcinoma cell lines with very different karyotypes. While this does not exclude the possibility that covert karyotypic abnormalities shared by the two cell lines were responsible for the observed abnormal development, it makes it less likely that the individual karyotypes were responsible and suggests, rather, that other characteristics are involved in determining the ability of embryonal carcinoma cells to participate in normal embryonic development.

Developmental potential of embryonal carcinoma cells. As was discussed in the introduction, it is not clear whether embryonal carcinoma cells are normal embryonic cells and only resemble neoplastic cells because the developmental controls which usually regulate their behavior are absent, or whether they are, in fact, neoplastically transformed and are similar to other neoplastic cells with embryonic characteristics. The results of this study project show that regardless of whether embryonal carcinoma cells are normal embryonic cells or embryo-like neoplastic cells, they require the assistance of a substantial number of normal inner cell mass cells to participate in the normal pattern of development.

One might argue that the NG-2, PSA-1, and LT1-2D aggregates did no

worse than the 129 embryonic ectoderm controls, nearly equaling or exceeding the level of normal development of these *in utero*, and thus the entire phenomenon might be explained by the ostensible developmental stage difference between embryonal carcinoma cells and cleavage stage embryos. As pointed out earlier, however, 129 embryonic ectoderm cells were able to form a complete, normal fetus. In contrast, embryonal carcinoma cells were never found to form even the major portion of a normal fetus. Furthermore, in all of the experiments involving embryonal carcinoma cells, regardless of the cell line used, there was a very clear correlation between the level of participation of the cells in the aggregates and the degree of abnormal development of the aggregates *in utero* (figure 14). Thus, despite the fact that the 129 embryonic ectoderm controls developed abnormally, and despite the fact that differences in embryonic stage also may have acted to disturb development, it seems clear that the embryonal carcinoma cells were not able to support development by themselves, once supplied with trophoblast.

These results suggest that normal inner cell mass cells exert a normalizing effect on embryonal carcinoma cells and that when this normalizing effect is not exerted strongly enough, development is abnormal. The fact that both PSA-1 and LT1-2D were found to require "normalization" is consistent with the possibility that this is a requirement of all embryonal carcinoma cells. Experiments with more cell lines would clarify this issue. The greater the number of cell lines shown to require inner cell mass cell "normalization", the lower the probability that these share a deficiency resulting from a secondary event in their culture history and not related *per se* to their identity

as embryonal carcinoma cells.

Embryonal carcinoma cell lines have been found to vary widely in their developmental capabilities (for review see Papaioannou, 1979). This is most likely due to secondary effects of the cell culture environment and to the history of the tumor from which the cell line was isolated. With this in mind, of particular interest would be experiments with cell lines, such as Mett-1 (Stewart and Mintz, 1981), that have formed functional germ cells in chimeric mice in previous studies and that have thus demonstrated full developmental potential. If these cells were found to require inner cell mass cells this would be strong evidence that embryonal carcinoma cells in general, even those which have shown totipotent developmental potential, require cycling through development in association with normal embryonic cells before they can express the full extent of their potential. (An intimation that Mett-1 requires ICM cells appears in the results of blastocyst injection experiments with these cells, see below.)

Other investigators have provided evidence that successful interaction between inner cell mass cells and embryonal carcinoma cells is critical for normal development. Illmensee (1978) described experiments in which inner cell masses were surgically removed from blastocysts and replaced with a group of 20 embryonal carcinoma cells. Although these implanted, they failed to develop and formed tumors when transplanted to a subcutaneous site. From this Illmensee concluded that the embryonal carcinoma cells required close association with normal embryonic cells for normalization. Papaioannou et al (1975, 1978) observed a high incidence of tumors in animals that developed from blastocysts injected

with embryonal carcinoma cells. Papaioannou (1979) has speculated that the tumors developed from embryonal carcinoma cells which were not normalized by the embryonic environment either because they were unable to respond to the embryonic cues due to secondary genetic changes *in vitro* or because communication between the embryo and the embryonal carcinoma cells was defective. Even Mett-1, a diploid cell line shown to have full developmental abilities (i.e. contribution to the germ line of a chimeric mouse), formed a tumor in one case in a chimeric mouse. As an explanation, Stewart and Mintz (1981) suggested that one of the cells injected into the blastocyst failed to integrate properly into the embryo.

It is interesting to note that in the above experiments, the improperly integrated or associated embryonal carcinoma cells formed tumors. Although one tumor was observed in this study, abnormal development was expressed primarily as retardation with some deformity in the more severely abnormal classes. Despite the fact that the cells were sufficiently well integrated to not form tumors, many of the embryos containing embryonal carcinoma cells still developed abnormally. From this it may be concluded that even apparent integration of the embryonal carcinoma cells into the embryo, accompanied by complete reversal of their neoplastic phenotype, is not sufficient for the normal development of a mouse from these cells.

Whether embryonal carcinoma cells are true neoplastic cells or not may be a question of semantics and cannot be resolved at this time. If embryonal carcinoma cells are normal embryonic cells they are not strictly equivalent to embryonic ectoderm cells since they have been

shown in these experiments not to have the developmental capabilities of embryonic ectoderm, i.e. they have not been able to form a normal midgestation fetus without the presence of large numbers of host SWR cells. It is possible that they may be equivalent to an as yet unidentified and more limited subset of embryonic ectoderm cells. If embryonal carcinoma cells are neoplastically transformed, then this transformation involves not only the expression of neoplastic growth but also a limitation in developmental potential which is reversible by the normal embryonic environment.

Development of Embryonal Carcinoma - Embryo Aggregates to Birth

In addition to assessing the developmental potential of embryonal carcinoma cells, this study examined the effectiveness of the aggregation technique for incorporating embryonal carcinoma cells into chimeric mice. In order to compare the results from incorporation by aggregation with the results from incorporation by injection, NG-2 - 2(ICRXSWR) aggregates were allowed to develop to term after transfer to pseudopregnant females. NG-2 - 2(ICRXSWR) aggregates were used because this appeared to be the optimal combination for successful incorporation of NG-2 into normal fetuses. Attention was focussed on the incorporation of NG-2 because results from injection of NG-2 into blastocysts had been published.

Frequency of implantation. The frequency of implantation and development of NG-2 - embryo aggregates to midgestation was 35%. This is similar to frequencies of implantation and development (30-40%) that

have been obtained both in this laboratory and by other investigators for aggregates composed totally of normal embryos (Bowman and McLaren, 1970; Mullen and Carter, 1973). Thus, the presence of embryonal carcinoma cells in the aggregates seems not to have adversely affected the frequency of implantation per se of the aggregates.

The frequency of development to birth, 13%, of the NG-2 - embryo aggregates was significantly lower than that of embryo - embryo aggregates. This decreased frequency of survival to birth was most certainly due to the high level of abnormal development of the NG-2 - embryo aggregates. As can be seen in table 4, the proportion of embryonal carcinoma cell - embryo aggregates that implanted was equivalent to that of normal embryo controls, however, the proportion of the aggregates that developed normally and that would have been expected to survive to term was much lower. This proportion of normal fetuses at midgestation is comparable to the proportion of mice actually born from aggregates allowed to develop to term in pseudopregnant females.

The frequency of survival to birth of NG-2 - embryo aggregates was lower than that reported for blastocysts injected with embryonal carcinoma cells. For example, 31% of the blastocysts injected with Mett-1 survived and developed to birth (Stewart and Mintz, 1981). (Frequencies of survival to birth were not reported for blastocyst injections utilizing NG-2.) Since there are no reports in the literature concerning the midgestation development of injected blastocysts in utero, it cannot be determined whether the higher frequency of survival to birth of injected blastocysts is due to a higher level of normal development in utero or whether injected blastocysts also have a high frequency of

abnormal development which is compensated for by a higher frequency of implantation and early development.

Two factors, removal of the zona pellucida and culture in vitro, that are necessary for aggregation of either embryonal carcinoma cells or normal embryos with embryos have been found to decrease development in utero. Most experiments report results in terms of survival to birth. However, since these experiments were done with embryo - embryo aggregates which would be expected to develop completely normally, development to birth is most likely a reflection of the frequency of successful implantation and early development. Removal of the zonae was found both by Mullen and Carter (1973) and by Bowman and McLaren (1970) to decrease development in utero. Mullen and Carter report that survival to birth decreased from 64.9% to 39.5% when zonaes were removed from the embryos. Bowman and McLaren report a less significant decrease. The frequency of survival of their embryos fell from 29.4% to 21.1% when zonaes were removed. In the same series of experiments, Bowman and McLaren found that after culture of the embryos in vitro for up to 29 hours their frequency of survival to birth decreased from 60% to 44%. Since blastocyst injections do not involve these experimental manipulations, injected blastocysts may, in general, develop better in utero. However, they are still subjected to a number of manipulations in vitro in addition to at least a short period of culture in vitro. The mechanical trauma of the injection process (indicated, for example, by the collapse of many blastocysts after injection) may also adversely affect implantation rates and early development.

Relevant to this discussion are the results of Gardner and Rossant

(1979) who injected embryonic cells from 4-1/2 day fetuses into blastocysts. Approximately 80% of these were found to have implanted and developed normally at 16 days post coitum. This frequency of implantation is significantly higher than the frequency of implantation and development obtained by Rossant (1975) in comparable experiments in which she aggregated inner cell masses of 3-1/2 day and 4-1/2 day fetuses with 8-cell embryos. The frequency of implantation and development of these embryos ranged from 13% to 49%. These results suggest that blastocysts which have been injected with embryonic cells have a better chance of implanting and developing than have embryos which have been aggregated with other embryos. It is likely that this is also true of blastocysts that have been injected with embryonal carcinoma cells.

Incidence of contribution. The incidence of contribution by NG-2 to the tissues of the developing aggregates was high, 62% (table 2, #+A/#tot), at midgestation. This figure dropped considerably, to 29% (7/24), during the last half of gestation partially because the abnormal aggregates died and were absorbed. Part of this loss also appeared to be due to a decrease in NG-2 participation in normal fetuses since the proportion of normal fetuses containing NG-2 derivatives at midgestation, 44%, was higher than the proportion at birth, 29% (table 4, #norm+A/#normal).

The survival of NG-2 derivatives in mice at birth after incorporation by aggregation compares well with the survival of embryonal carcinoma cells injected into blastocysts. Embryonal carcinoma cells derived directly from ascites tumors (OTT 6050) in two different studies survived at a frequency of 13.9% and 25.3% in animals at birth (reviewed

by Illmensee, 1978). The diploid cell line Mett-1 survived at a frequency of 13%. This number may have been somewhat higher since only animals with coat mosaicism were extensively assayed for embryonal carcinoma cell contribution. Of the other animals analyzed, 16.6% (2/12) of the neonates (analyzed before their coats had developed) and 27% (4/15) of the animals without coat mosaicism contained embryonal carcinoma cell derivatives (Stewart and Mintz, 1981).

In the experiments in which NG-2 cells were injected into blastocysts, only 12 of the 44 animals obtained were analyzed for the presence of NG-2 derivatives (Dewey et al, 1977). Of these, 9 (75%) contained NG-2 derivatives. In the past five years, no information regarding the remaining 32 mice has been published. If these are assumed not to have been chimeric, then the overall incidence of NG-2 derivatives in these mice falls to 23%. This is more comparable to the incidence found in blastocyst injections of other embryonal carcinoma cells and to the incidence found in aggregations of NG-2.

Extent of contribution. Contribution by NG-2 derivatives to normal tissues was sporadic, varying from one to eight tissues. This is in agreement with the pattern of contribution found in animals derived from blastocysts injected with embryonal carcinoma cells. In the blastocyst injection experiments with Mett-1, for example, 17 of the 38 mice with coat mosaicism had embryonal carcinoma cell derivatives in only their coats and in no other tissues. NG-2, when injected into blastocysts, contributed to more than four tissues in only 4 out of the 9 chimeric mice. It is also true, however, that 2 of the above 4 mice contained NG-

2 derivatives in a broad range of 13 and 14 tissues. This is more extensive than the distribution of derivatives in mice developed from aggregates. In these four mice the number of tissues containing NG-2 derivatives ranged from four to eight. This may reflect the fact that aggregates in which NG-2 made major contributions developed abnormally and died in utero and only those with relatively minor contributions at the outset survived. The substantial variation found among chimeric mice in the extent of embryonal carcinoma cell incorporation into their tissues, however, also makes it possible that the foregoing observations merely reflect one of the many random variations found in small samples.

Examination of the tissues to which NG-2 made substantial contributions (table 5) reveals that in both aggregation experiments and blastocyst injection experiments NG-2 contributed most heavily to the heart. The pattern of distribution of NG-2 derivatives among the other tissues analyzed was also similar, with the exception of brain and spleen. NG-2 contributions to these were conspicuously missing in animals developed from aggregates whereas NG-2 contributions were often present, although at low levels, in these tissues in animals developed from injected blastocysts. It remains to be seen whether this trend will continue as more animals developed from aggregates are analyzed.

With regard to the germ line, only one animal contained NG-2 derivatives in its gonads and this contribution was small. Even if NG-2 were to make substantial contributions to the gonads of chimeric mice, the chromosomal abnormalities of the cells (trisomy 6, XO) make it unlikely that they would form functional germ cells. Although trisomy 21 does not prevent functional germ cell formation in humans (Penrose and

Smith, 1966) most of the work with mice suggests that trisomies interfere with spermatogenesis, if not oogenesis. Cattanach (1967) found that spermatogenesis was defective in an adult mouse trisomic for chromosome 16 and Griffen and Bunker (1964) found that three trisomic offspring from an irradiated male were sterile or semi-sterile. There is some doubt regarding the identification of the trisomies in the above experiments since chromosome size was the only method of identification used. It is possible that the third chromosomes in the trisomic sets were actually small translocation products (Lyon and Meredith, 1966). Nevertheless, in agreement with the above experiments, Cattanach (1967) has also found sterility in a mouse partially trisomic for chromosome 6 although the mouse was otherwise phenotypically normal.

The absence of a second sex chromosome is less damaging to the germ cell-forming potential of NG-2. In mice, XO females are fertile although they tend to produce smaller litters (Cattanach, 1962; Lyon and Hawker, 1973). There is evidence that the "O" is preferentially lost into the polar body (Kaufman, 1972). In male mice, however, XO germ cell formation is less likely. It has been found that spermatozoa formed in an $X^{sxr}O$ testes exhibit little or no motility (Cattanach et al, 1971).

It is unfortunate that both of the above discussed chromosomal abnormalities adversely affect spermatogenesis in particular since the majority of the mice developed from NG-2 - 2(ICRXSWR) aggregates (16/24) were phenotypically male. This is as would be expected from the combination of two embryos since half of the offspring from such a combination would, theoretically, be intersex chimeras and since intersex chimeras are known to be most often phenotypically male in balanced

strain combinations (Mullen and Whitten, 1971). This disadvantage will not necessarily exist in all cases in which aggregation is used to incorporate embryonal carcinoma cells into chimeric mice. The experiments with PSA-1 demonstrate that, with the appropriate cell lines, it is quite possible to obtain reasonably frequent incorporation of embryonal carcinoma cells into normal mice by using only one embryo in the aggregation. Thus, there is no reason to doubt that germ line integration by capable embryonal carcinoma cells will be as likely to occur in chimeric mice generated by aggregation of the cells with embryos as in those generated by injection of the cells into blastocysts.

Aggregation compared with blastocyst injection. In conclusion, as a technique for incorporating embryonal carcinoma cells into mice, aggregation with cleavage-stage embryos has advantages and disadvantages which may balance each other when compared to the blastocyst injection technique. Initial incorporation of embryonal carcinoma cells into embryos by aggregation is very high. Unfortunately this often results in abnormal development and death in utero. This is partially due to the developmental problems that occur when cells of different embryonic stages are combined. However, this is also related to the potential of the embryonal carcinoma cells and cells with better potential, such as Mett-1, might support more normal development. In this case, aggregation would be a very effective method for incorporating large numbers of embryonal carcinoma cells into mice. Since no midgestation data are available regarding the in utero development of injected blastocysts, it is not known whether many of these also contain a large proportion of

embryonal carcinoma cells, develop abnormally, and die before birth. Since the frequency of survival to birth of blastocysts injected with embryonal carcinoma cells has been found to be lower than that of blastocysts injected with normal embryonic cells, it is quite possible that a significant number die *in utero*. Whether this is due to abnormal development is unclear.

The initial ratio of normal embryonic cells to embryonal carcinoma cells is critical when aggregation is used to incorporate cells into embryos. Development at midgestation must be examined to be sure that the embryonal carcinoma cells are not dominating the aggregates to an extent that prevents survival to birth of a significant number of them. Despite this complication, the results of this study show that aggregation of embryonal carcinoma cells with embryos results in incorporation of the cells into liveborn mice which is comparable, both in incidence and extent, with the incorporation obtained by injecting the cells into blastocysts. Since survival to birth is lower, it is necessary to do more aggregations than injections to obtain a comparable number of chimeric mice. However, this is easily done since, relative to blastocyst injections, less technical expertise is required and fewer specialized pipets need to be manufactured. Furthermore, the necessary equipment is much less expensive and more commonly available.

Conclusion

As stated in the introduction, this study was aimed at examining the developmental potential of embryonal carcinoma cells. Concomitantly, aggregation with cleavage-stage embryos was explored as a method for

more effectively incorporating embryonal carcinoma cells into mice.

Embryonal carcinoma cells were found to be fully capable of forming the entire inner cell mass of a blastocyst. For further development, however, they required the presence, and ostensibly the assistance, of normal inner cell mass cells. This was established by the fact that composite embryos containing a large proportion of embryonal carcinoma cells developed abnormally. Thus, these embryonal carcinoma cells were more limited in their ability to direct normal development than either inner cell mass cells or embryonic ectoderm cells were found to be. Since all of these cell lines were karyotypically abnormal, it is not clear whether this limitation is due to a shared chromosomal defect or whether it is an intrinsic limitation shared by all embryonal carcinoma cells. Experiments with totipotent cells, i.e. cells such as Mett-1 which have contributed to the germ line, would help to determine this.

As a technique for incorporating embryonal carcinoma cells into mice, aggregation with cleavage-stage embryos appeared to be as effective as injection of the cells into blastocysts. Although the initial ratio of normal embryo cells to embryonal carcinoma cells is critical and effort must be expended to determine the optimum combination, and although survival to term is lower than that of injected blastocysts, these disadvantages may be compensated for by the greater ease with which the manipulations may be learned and the fact that expensive, specialized pieces of equipment are not required.

Figure 1. Diagram of experimental procedure.

SWR/J mice were mated and eight-cell stage embryos were collected from the pregnant females. Embryonal carcinoma cells were induced to form small clumps by growing them in bacteriological petri dishes. Embryos were aggregated with clumps of embryonal carcinoma cells in the presence of PHA, removed from the PHA, rinsed, and cultured for 24 hours. Blastocysts were obtained for glucose phosphate isomerase (GPI) assays by culturing the aggregates for an additional 24 hours (A). Alternatively, the compacted aggregates were transferred to the uteri of pseudopregnant females. Midgestation fetuses were studied by sacrificing recipient foster mothers eight days after transfer of the aggregates to their uteri. All implants were dissected away from the decidua, rinsed in Phosphate buffered saline, photographed, and analyzed for GPI phenotype (B). Liveborn mice were obtained by allowing the foster mothers to carry their litters to term. Approximately seven to ten days after birth, the mice were examined for coat mosaicism. Those not displaying agouti hairs in their coat were sacrificed and fifteen tissue samples were collected for GPI analysis (C).

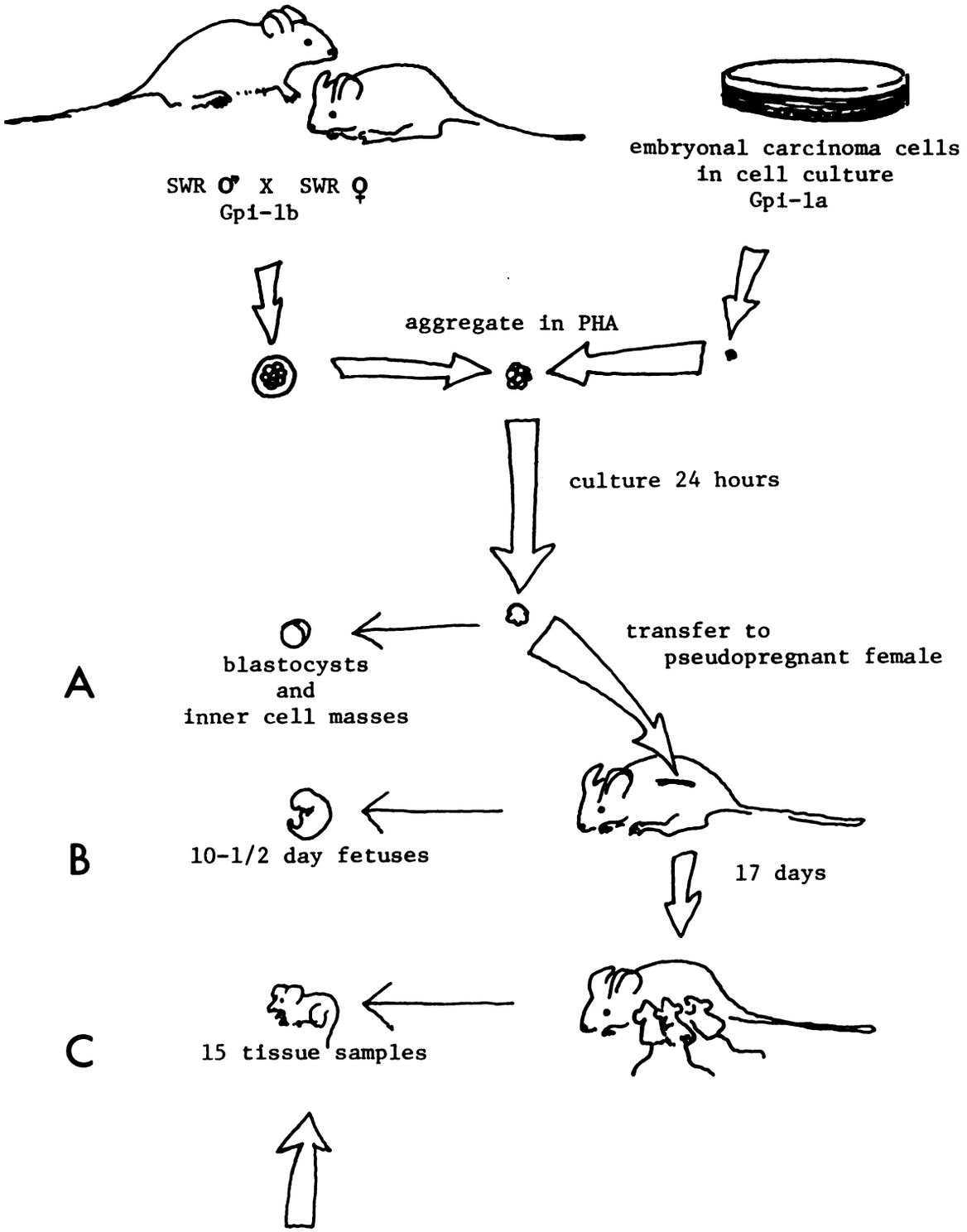

Figure 1. Diagram of experimental procedure.

Figure 2. Aggregation of embryonal carcinoma cells with one, split eight-cell stage embryo.

A. Two sets of four blastomeres each from the same SWR/J embryo were arranged around a clump of PSA-1 embryonal carcinoma cells (ecc). B. Within 24 hours of aggregation, the aggregate had compacted to form an apparently normal morulae. C. Within 48 hours, the aggregate had formed an apparently normal blastocyst with an inner cell mass (icm) surrounded by trophoblast (t). Magnification 300X.

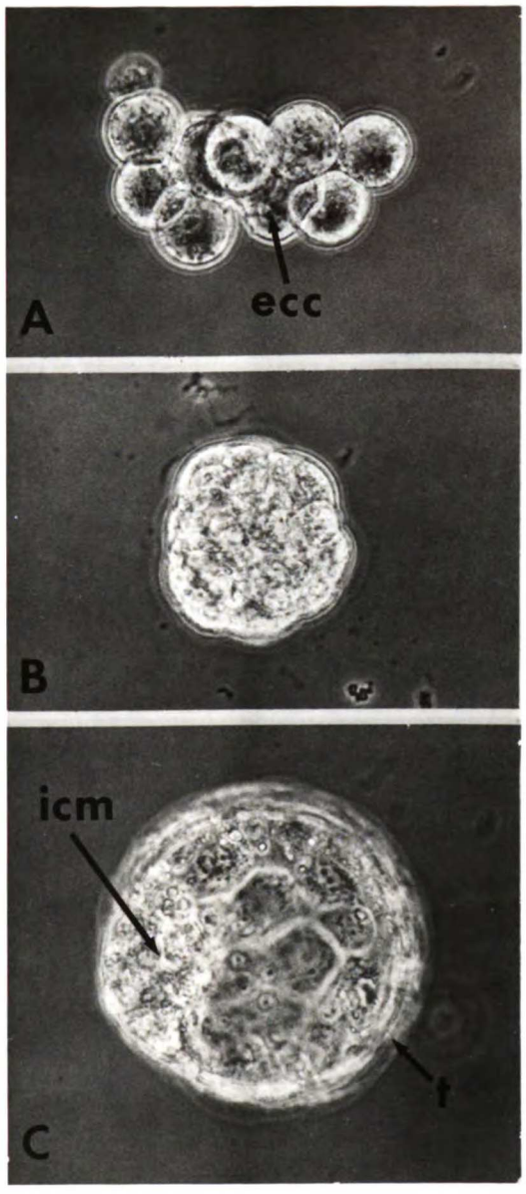

Figure 2. Aggregation of embryonal carcinoma cells with one, split eight-cell stage embryo.

Figure 3. Aggregation of PYS and STO cells with split, eight-cell stage embryos.

A. Blastomeres from an eight-cell stage SWR/J embryo were arranged around a clump of PYS cells in the presence of PHA. B. Within a few hours after removal from PHA the aggregate fell apart. C. Subsequently, the PYS cells attached to the plate and grew out. D. Similarly, eight-cell stage blastomeres were arranged around a clump of STO cells. E. After removal from PHA the aggregate fell apart. F. Within a few hours the STO cells also attached to the plate and grew out. Magnification 300X.

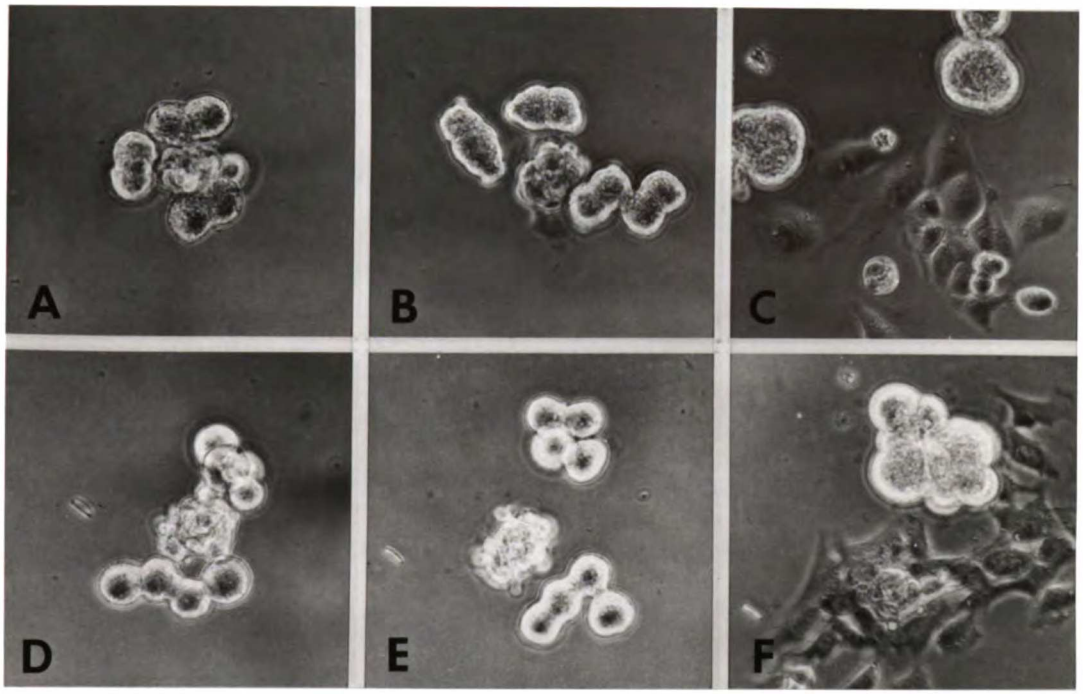

Figure 3. Aggregation of PYS and STO cells with split, eight-cell stage embryos.

Figure 4. GPI analysis of blastocysts and inner cell masses resulting from the aggregation of PSA-1 embryonal carcinoma cells with split, eight-cell stage, SWR/J embryos.

a. Control Gpi-1a isozyme, embryonal carcinoma cell homogenate. b. Control Gpi-1b isozyme, SWR/J liver homogenate. c. and d. Four and two blastocysts, respectively, developed from aggregates composed of clumps of PSA-1 embryonal carcinoma cells surrounded by SWR/J eight-cell stage blastomeres. e. A group of five inner cell masses isolated from blastocysts similar to those in c and d. Note the presence of only one band, corresponding to the embryonal carcinoma Gpi-1a isozyme.

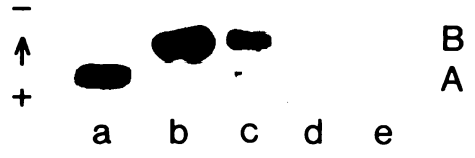

Figure 4. GPI analysis of blastocysts and inner cell masses resulting from the aggregation of PSA-1 embryonal carcinoma cells with split, eight-cell stage, SWR/J embryos.

Figure 5. Aggregation of embryonal carcinoma cells with one, intact, eight-cell stage embryo.

A. One, intact, eight-cell stage SWR/J embryo was aggregated with a clump of NG-2 embryonal carcinoma cells in the presence of PHA. B. Within 24 hours of aggregation, the aggregate had compacted to form an apparently normal morula. C. Within 48 hours, the aggregate had formed an apparently normal blastocyst. Magnification 300X.

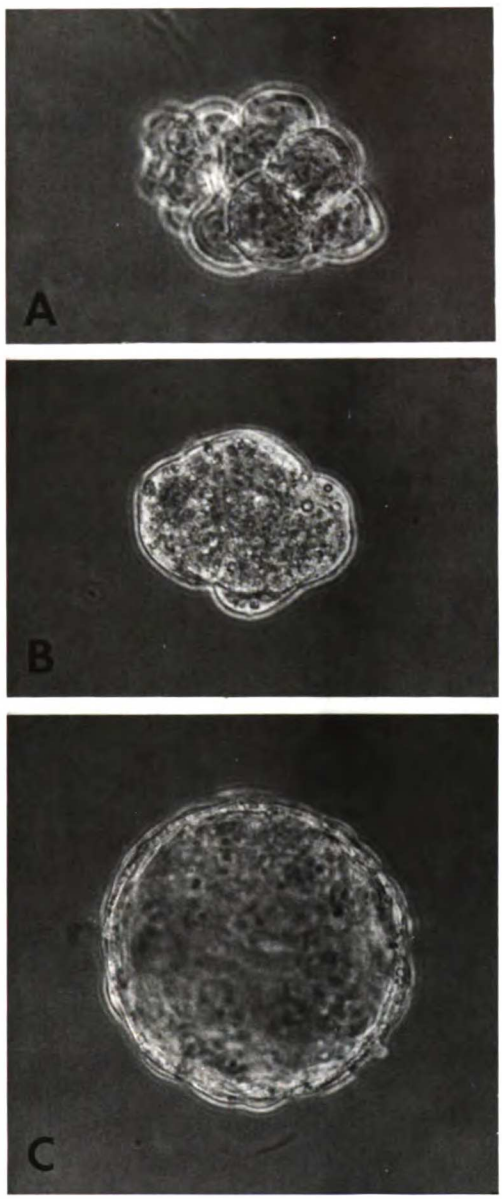

Figure 5. Aggregation of embryonal carcinoma cells with one, intact, eight-cell stage embryo.

Figure 6. Abnormal phenotypes found at midgestation.

Abnormal phenotypes found at midgestation arranged from the least abnormal in the upper left corner (6) to the most abnormal in the lower right corner (1). Numbers refer to the classification group.

Group 6 fetuses closely resemble normal 10-1/2 day midgestation fetuses. However, note the abnormally shaped head in this sample. Group 5 fetuses resemble retarded 9-1/2 day fetuses. The sample on the right also appears slightly deformed with an expanded heart sac and oddly formed mandibular arches. Group 4 fetuses appear much more retarded. Note, however, the closed appearance of their body cavities and the developing hearts (arrows) protruding from the ventral side just under the head region. Group 3 fetuses are even more retarded. The sample on the right has been dissected away from the amnion and the yolk sac. Developing somites (arrow) can be seen on either side of the open neural tube. Group 2 fetuses are small and flat. Their structures are usually hidden by the enclosing amnion, as on the left. When the amnion is dissected away, as on the right, enlarged head folds can be seen on one end while an allantoic stalk usually protrudes from the other. Sometimes a crooked neural groove can be seen extending the central length of the fetus. Group 1 samples are usually lumps as seen on the right. However, sometimes disorganized structures are arranged on the yolk sac as seen on the left. Note the large, deformed cephalic-like structures on the lower right. A heart tube (arrow) is faintly visible just above and to the left of the head structure. The structure in the lower left corner resembles an allantois. For a complete description of the classification groups see Results. Magnifications: 6 = 12X; 5, 4, 3, 2, 1 = 25X.

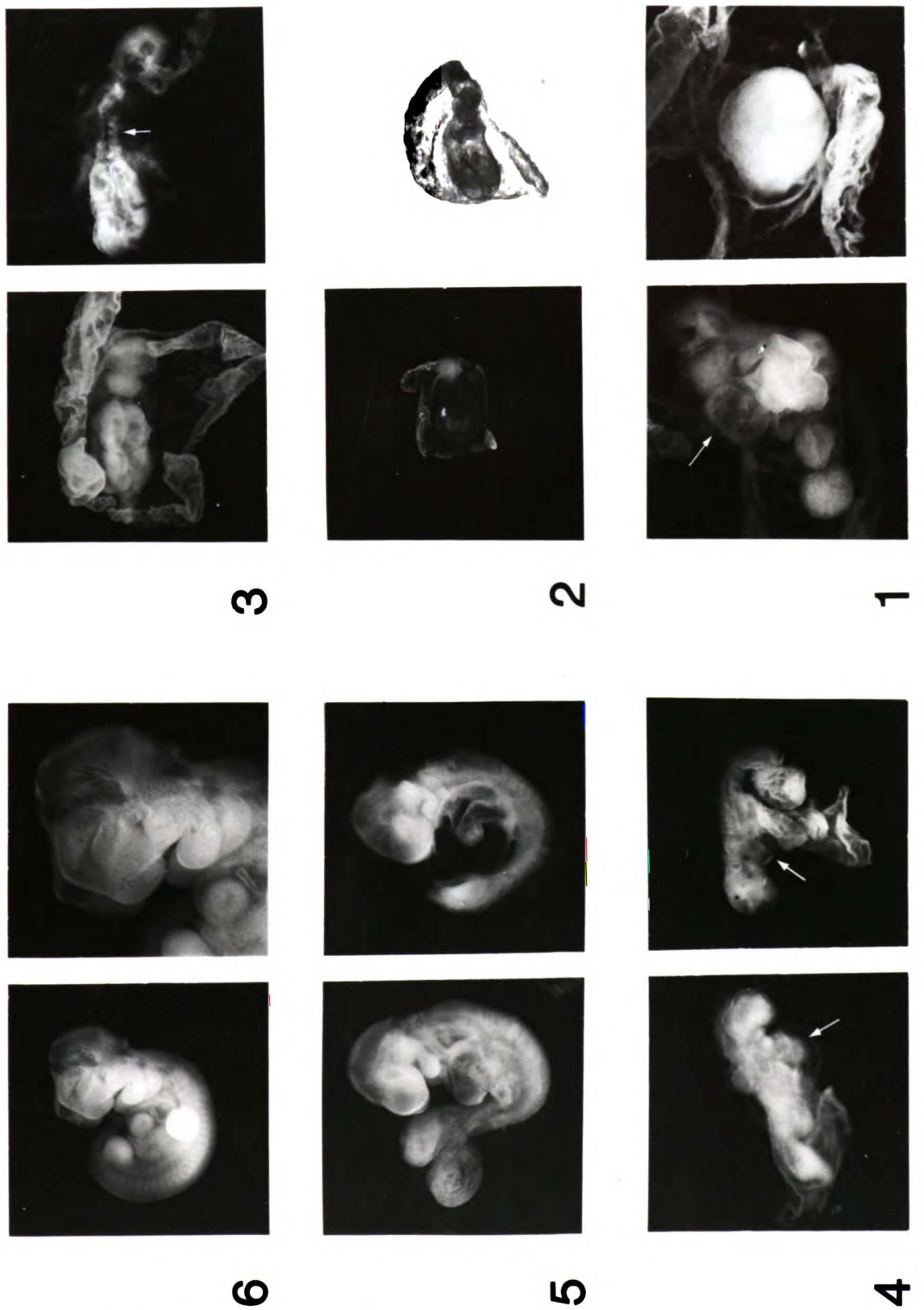

Figure 6. Abnormal phenotypes found at midgestation.

Figure 7. Sections of a fixed and embedded group 3 fetus.

The group 3 fetus shown in B was fixed and embedded and two micron horizontal sections were cut. A. shows a section from the cephalic end (left side of picture B, upper arrow). Comparison with the diagram on the right of a section through a normal 8-1/2 day fetus reveals that the structures seen in A resemble those of a normal fetus. C. shows a section taken from a region caudal to A (lower arrow) and, similarly, resembles the normal structures diagrammed on the right. Diagrams from Rugh (1968), p.110 and 111. Magnifications: A, C = 182X; B = 25X.

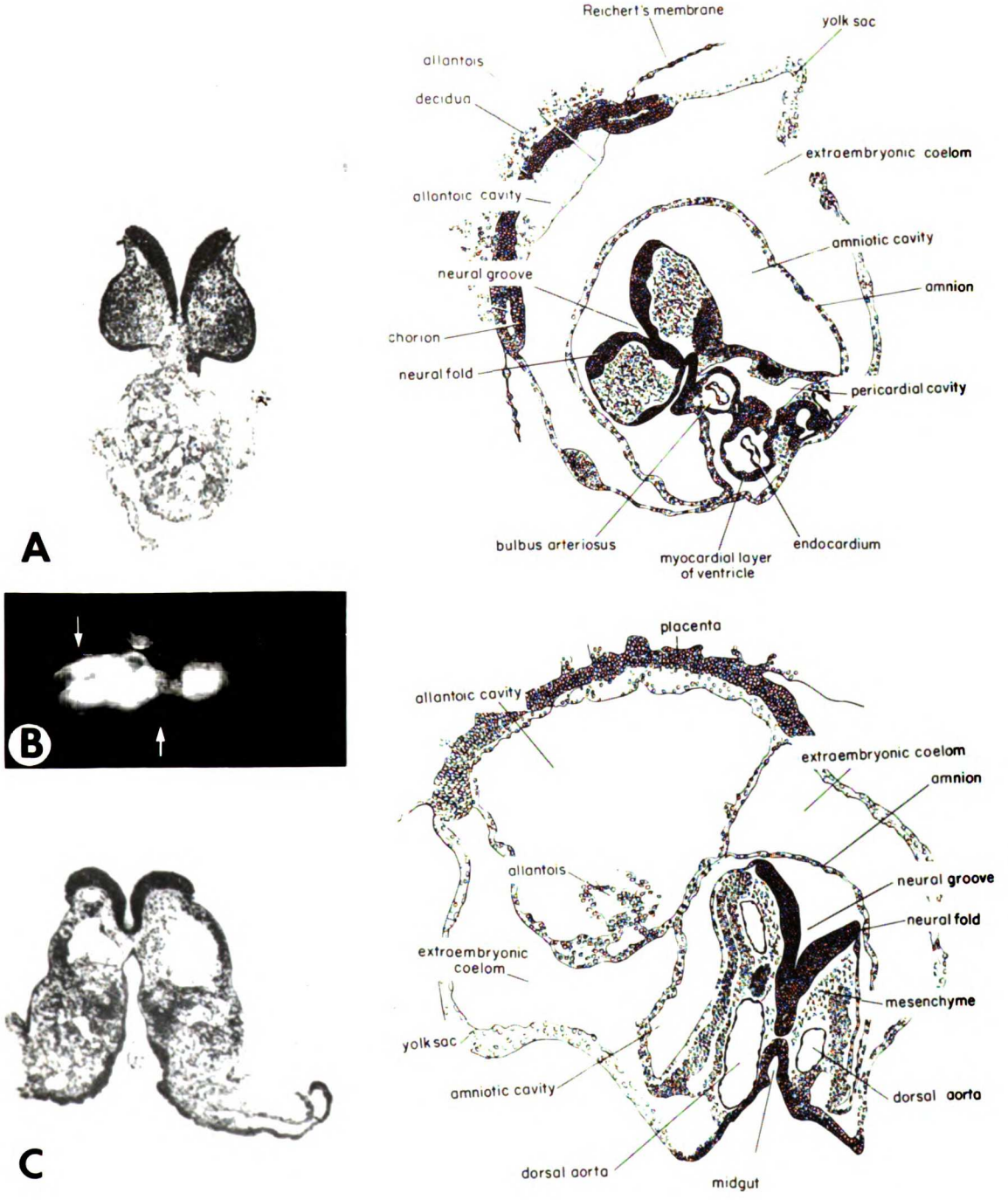

Figure 7. Sections of a fixed and embedded group 3 fetus.

Figure 8. Sections of a fixed and embedded group 2 fetus.

The group 2 fetus shown in B was fixed and embedded and two micron sagittal sections were cut. As can be seen in the sagittal section shown in A and in the enlargement shown in E, no groups of cells could be identified as misplaced, unintegrated embryonal carcinoma cells. Evidence that embronal carcinoma cells were probably present in B is illustrated in C and D. C is a sample similar to B. Note their close morphological similarity. D. is the GPI analysis of the sample shown in C and reveals that both the fetus proper and the membranes contained embryonal carcinoma derivatives as indicated by the presence of the slower migrating Gpi-1a isozyme band on the right, labelled A. Magnifications: A = 182X; B,C = 25X; E = 600X.

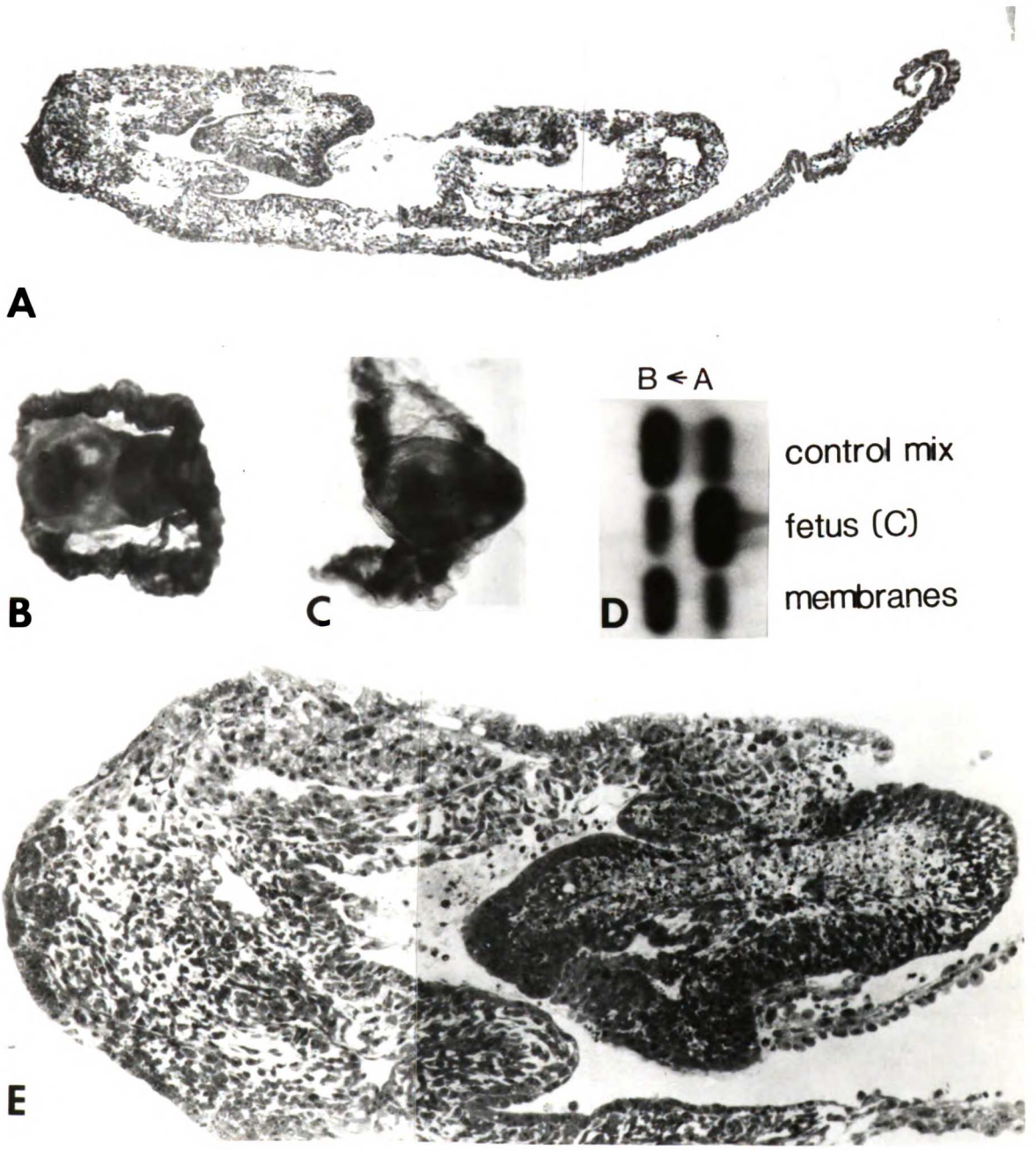

Figure 8. Sections of a fixed and embedded group 2 fetus.

Figure 9. Examples of commonly observed GPI isozyme ratios.

Samples analyzed electrophoretically for GPI isozymes were classified according to the ratio of the two isozymes found to be present. c, d and k represent samples in which only the Gpi-1b isozyme was present (all B). In a, h, i, l, m, and p the Gpi-1b isozyme was present in greater quantities than the Gpi-1a isozyme (B>A) whereas in o the two isozymes appeared to be present in roughly equal amounts (B=A). g, j and n are samples in which the Gpi-1a isozyme was present in greater quantities than the Gpi-1b isozyme (B<A). In e only the Gpi-1a isozyme was detectable (all A). b and f illustrate the pattern obtained from samples that are heterozygous for the two GPI isozymes, in this case, blood samples from foster mothers. Note that the three-banded pattern of a heterozygote is distinct from the two-banded pattern of a chimera.

Figure 9. Examples of commonly observed GPI isozyme ratios.

Figure 10. Control embryo - embryo aggregations: Normalcy of development versus extent of contribution.

Normalcy of development, as estimated by classification in one of the phenotypic groups described in the Results (N, normal, and 6 through 1), was plotted against the extent of contribution of the 129 cells as estimated by the relative amount of Gpi-1a isozyme present. "All B" indicates that no 129 derivatives were present whereas "all A" indicates that only 129 derivatives were present. Each symbol represents one midgestation sample.

Note that the samples tend to be distributed in the all B or all A categories. Most of the samples with abnormal phenotypes were from aggregations of eight-cell embryos with either 129 ICMs or 129 embryonic ectoderm.

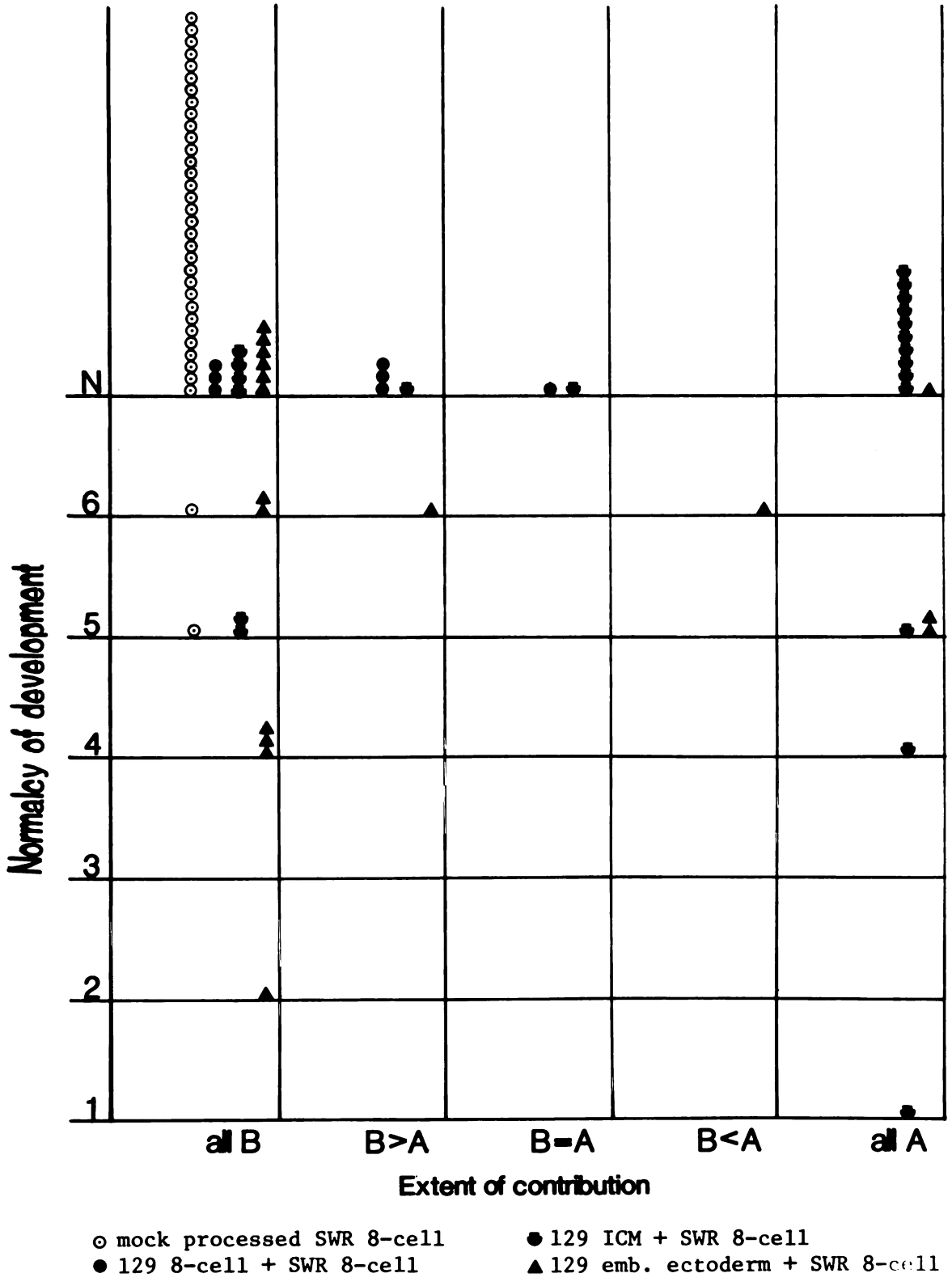

Figure 10. Control embryo - embryo aggregations: Normalcy of development versus extent of contribution.

Figure 11. NG-2 variations: Normalcy of development versus extent of contribution.

Normalcy of development was estimated by classification in one of the phenotypic groups described in the Results (N, normal, and 6 through 1). Extent of contribution was estimated by the relative amount of Gpi-1a isozyme present. "All A" indicates that only NG-2 derivatives were present, "all B" indicates that no NG-2 derivatives were present. Each symbol represents one midgestation sample.

Note that there is an inverse relationship between normalcy of development and extent of contribution. As the extent of contribution by NG-2 derivatives increases, normalcy of development decreases.

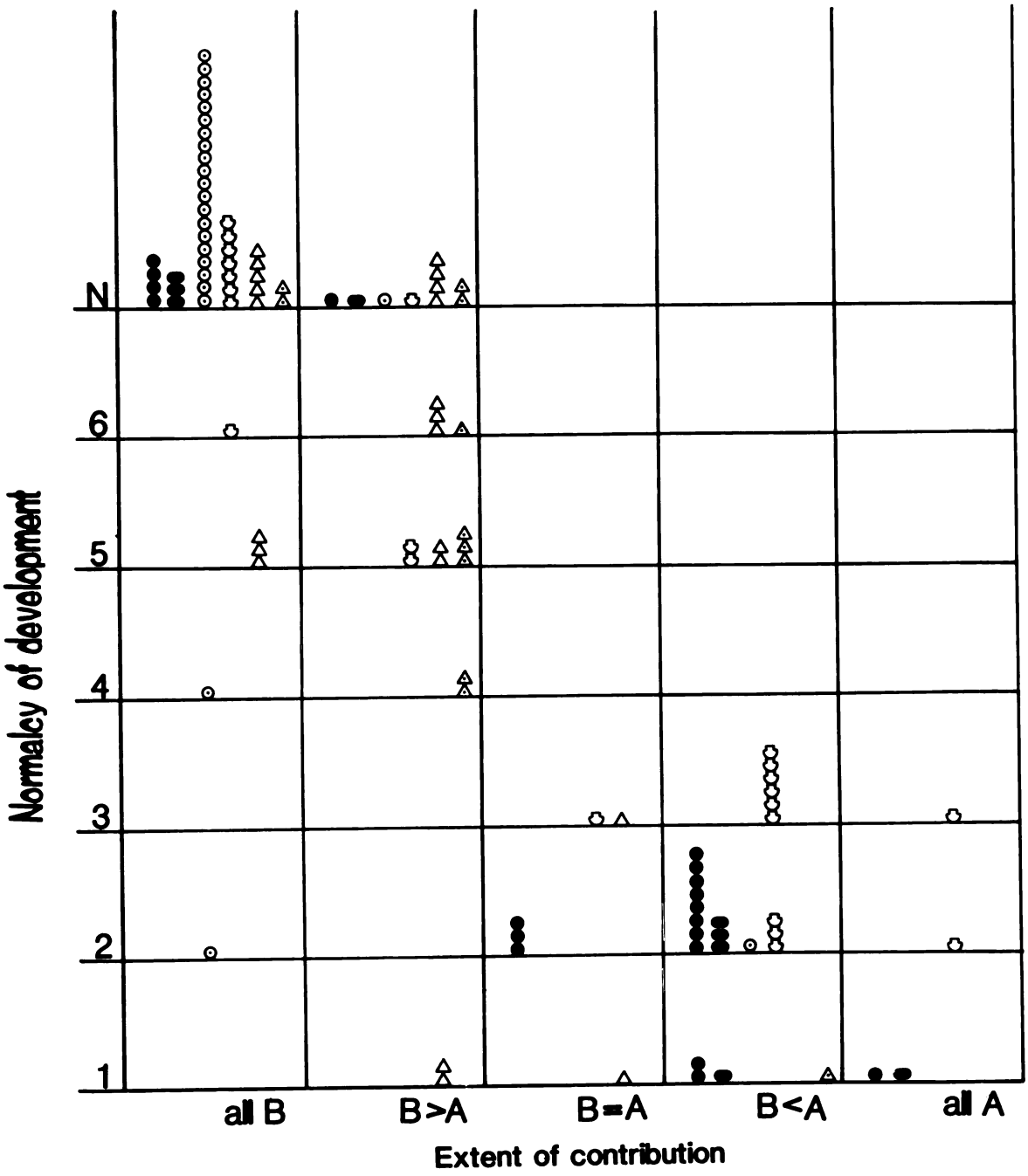

Figure 11. NG-2 variations: Normalcy of development versus extent of contribution.

Figure 12. Number of cells in embryonal carcinoma cell clumps used in aggregations.

Spreads of embryonal carcinoma cell clumps were prepared, stained with Giemsa stain, and the number of nuclei counted. A is a group of clumps approximately the size of one eight-cell stage blastomere. Most of the embryonal carcinoma cell clumps used in this study were of this size range. B is a group of smaller clumps. This size clump was used in the experiments which attempted to decrease the number of embryonal carcinoma cells in the aggregates as described in the Results. Note that although the average number of cells in group B (6) was less than the average number of cells in group A (10), there was a significant amount of overlap. This overlap may account for the fact that little difference in development was seen when aggregations were done with smaller clumps.

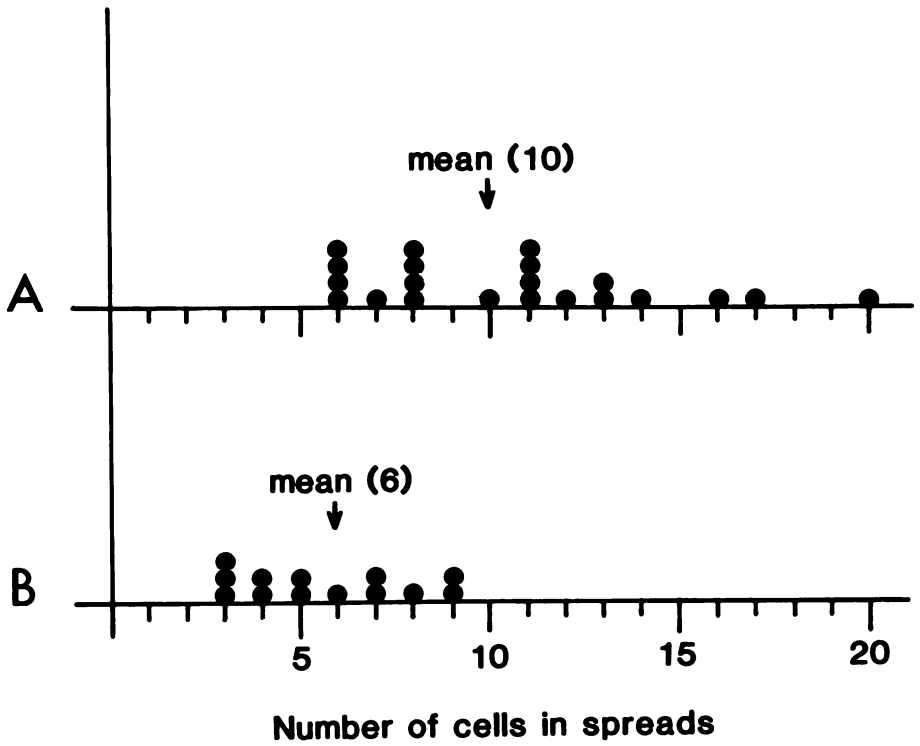

Figure 12. Number of cells in embryonal carcinoma cell clumps used in aggregations.

Figure 13. Aggregation of embryonal carcinoma cells with two, eight-cell stage embryos.

A. Two eight-cell stage embryos were arranged around a clump of embryonal carcinoma cells. B. Within 24 hours the aggregate had compacted to form an apparently normal morulae. C. Within 48 hours the aggregate had formed an apparently normal blastocyst. Magnification 300X.

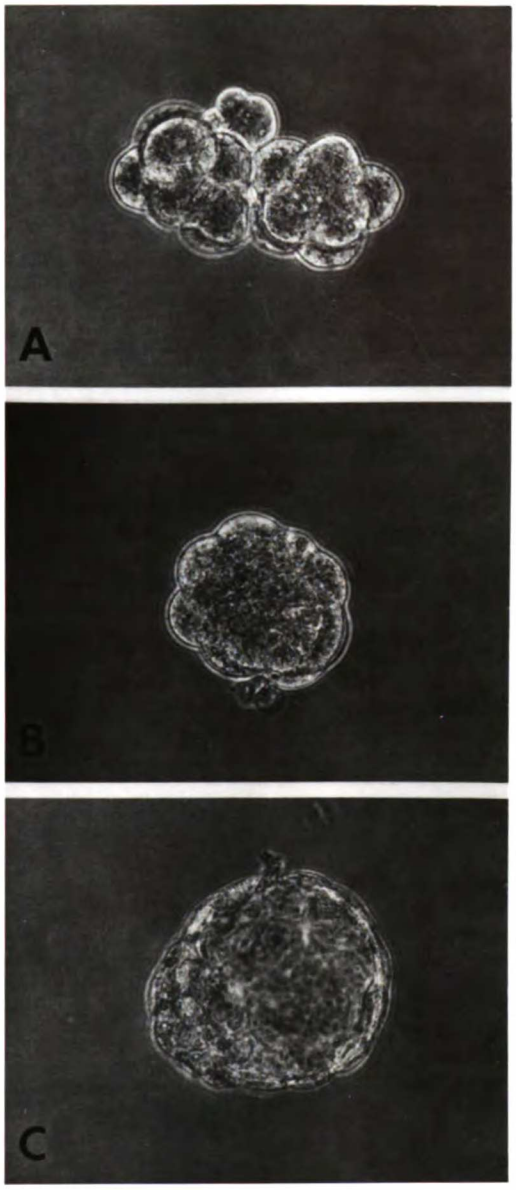

Figure 13. Aggregation of embryonal carcinoma cells with two, eight-cell stage embryos.

Figure 14. NG-2, PSA-1, and LT1-2D comparison: Normalcy of development versus extent of contribution.

Normalcy of development was estimated by classification in one of the phenotypic groups described in the Results (N, normal, and 6 through 1). Extent of contribution was estimated by the relative amount of Gpi-1a isozyme present. "All A" indicates that only embryonal carcinoma cell derivatives were present, "all B" indicates that no embryonal carcinoma cell derivatives were present. Each symbol represents one sample taken at midgestation.

Again, the data points suggest an inverse relationship between normalcy of development and extent of contribution by the embryonal carcinoma cells.

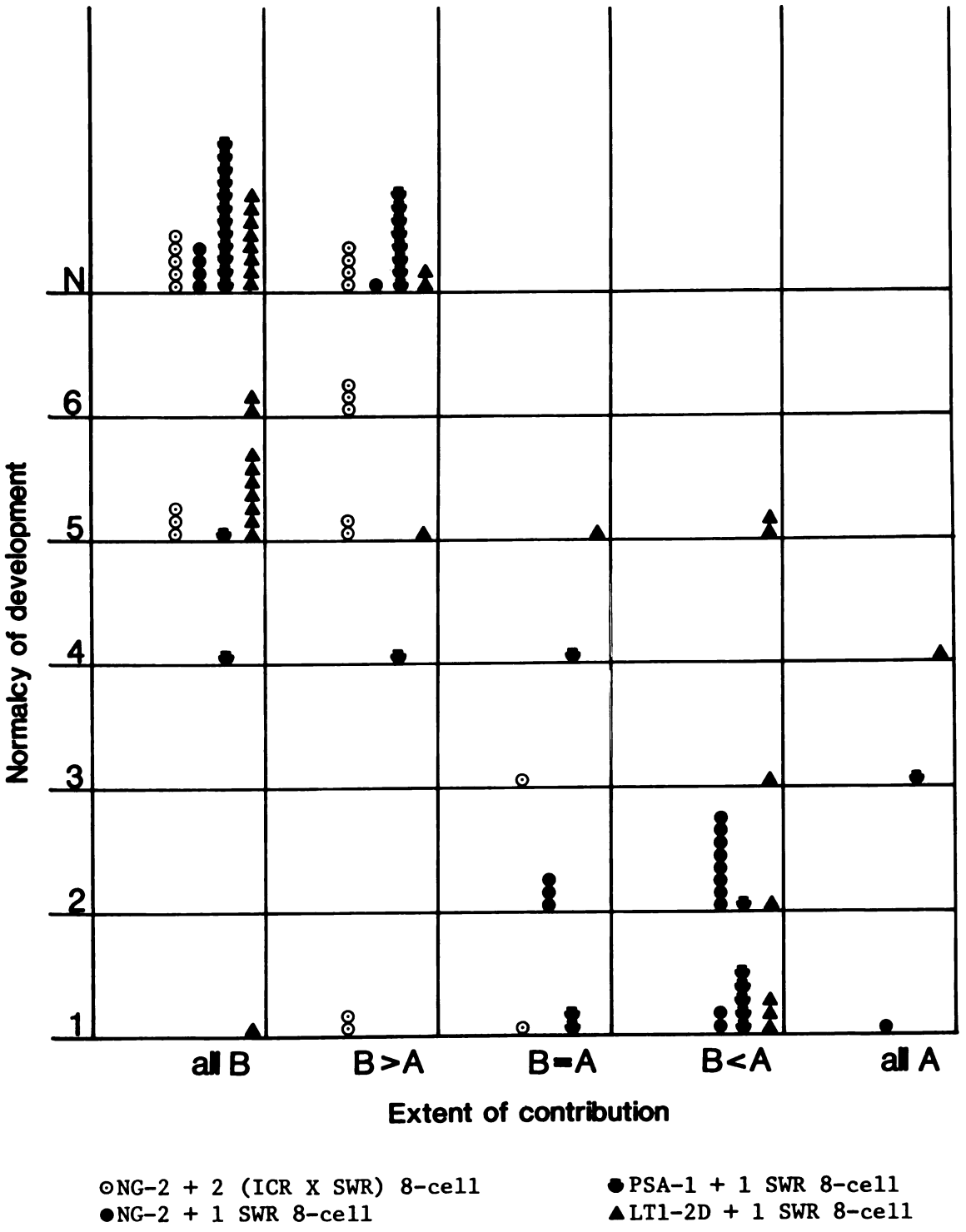

Figure 14. NG-2, PSA-1, and LT1-2D comparison: Normalcy of development versus extent of contribution.

Figure 15. GPI analysis of tissues from a chimeric mouse.

GPI analysis of tissues from an albino mouse resulting from the aggregation of two ICR X SWR embryos with a clump of NG-2 cells. a. heart, b. reproductive tract (excluding gonads), c. bladder, d. kidney, e. gut (stomach), f. pancreas, g. spleen, h. liver, i. control mix.

Note the presence of the slower Gpi-1a band, indicating the presence of NG-2 derivatives, in several tissues.

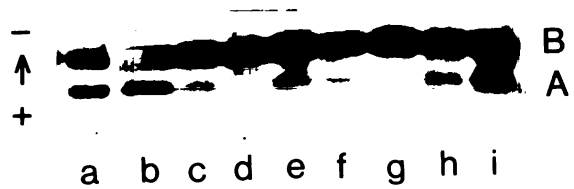

Figure 15. GPI analysis of tissues from a chimeric mouse.

Figure 16. "Spot II"

This male mouse was born to a female who had had NG-2 - 2(ICRXSWR) aggregates surgically transferred to her uteri. NG-2 contributions to the composition of his coat are visible in the patch of agouti hairs on the right side of his face (A) and the agouti stripe over his right shoulder (B).

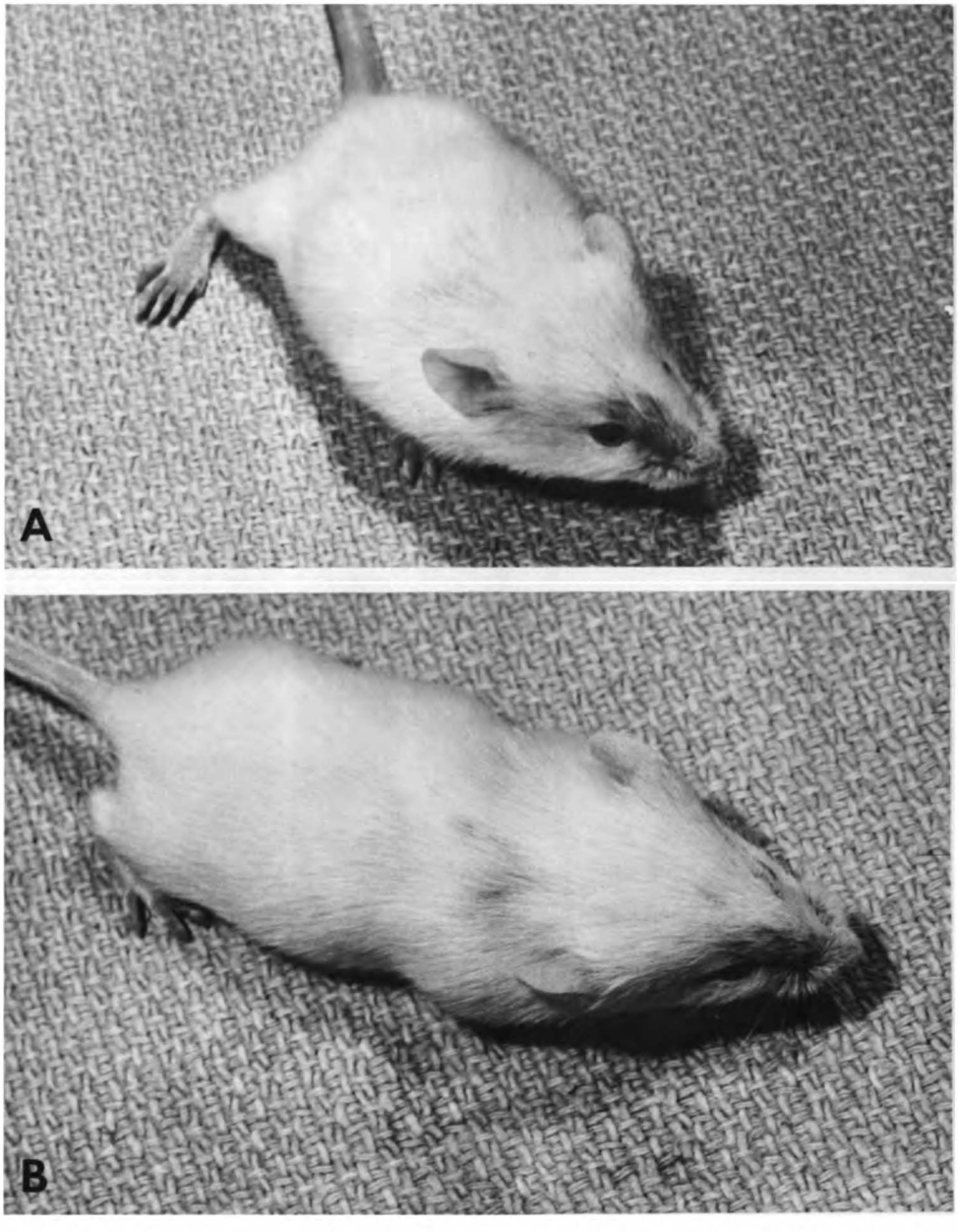

Figure 16. "Spot II"

Table 1. Phenotypes at midgestation

Phenotypes observed at midgestation are tabulated in two groups: Table 1A, those samples that contained embryonal carcinoma cell or control embryo cell derivatives as indicated by the presence of the Gpi-1a isozyme (+A) and Table 1B, those samples that contained no derivatives as indicated by the absence of the Gpi-1a isozyme (all B). The number of samples with (#+ A) and the number of samples without (# all B) derivatives are given in the first column on the left. N indicates a normal phenotype, 6 through 1 each indicate one of the phenotypic classifications described in the Results. Note that the farther left the samples are in the columns, the less abnormal and thus more normal are their phenotypes. Exp # refers to the number of experiments conducted to collect the given data.

Table 1A. Phenotypes at midgestation

Type of Aggregation	# +A	+A (all A)*							exp
		N	6	5	4	3	2	1	#
mock processed 8-cell	0								4
129 8-cell + 1 SWR 8-cell	4	4							1
129 ICM + 1 SWR 8-cell	15	2(10)*		(1)*	(1)*			(1)*	5
129 emb ecto + 1 SWR 8-cell	5	(1)*	2			(2)*			6
NG-2 + 1 SWR 8-cell	15	1					11	3	3
small NG-2 + 1 SWR 8-cell	6	1					3	2	3
NG-2 doub/trip + 1 SWR 8-cell	2	1					1		4
NG-2 + 2 SWR 8-cell	15	1		2		8	4		3
NG-2 + 2(ICRXSWR) 8-cell	13	4	3	2		1		3	2
NG-2 + 3(ICRXSWR) 8-cell	9	2	1	3	2			1	1
NG-2 + 1(ICRXSWR) 8-cell	2		1		1				3
PSA-1 + 1 SWR 8-cell	19	8			2	(1)*	1	7	3
LT1-2D + 1 SWR 8-cell	12	2		4	(1)*	1	1	3	5
NG-2 + 1 SWR 8-cell	15	1					11	3	3

* Numbers in parenthesis refer to samples containing only the GPI-1A isozyme.

Table 1B. Phenotypes at midgestation continued

Type of Aggregation	# all B	all B						
		N	6	5	4	3	2	1
mock processed 8-cell	34	32	1	1				
129 8-cell + 1 SWR 8-cell	3	3						
129 ICM + 1 SWR 8-cell	6	4		2				
129 emb ecto + 1 SWR 8-cell	12	6	2		3		1	
NG-2 + 1 SWR 8-cell	4	4						
small NG-2 + 1 SWR 8-cell	3	3						
NG-2 doub/trip + 1 SWR 8-cell	22	20			1		1	
NG-2 + 2 SWR 8-cell	8	7	1					
NG-2 + 2(ICRXSWR)	8	5		3				
NG-2 + 3(ICRXSWR)	2	2						
NG-2 + 1(ICRXSWR) 8-cell	12	12						
PSA-1 + 1 SWR 8-cell	14	12		1	1			
LT1-2D + 1 SWR 8-cell	18	8	2	7				1
NG-2 + 1 SWR 8-cell	4	4						

Table 2. Incidence and extent of contribution to midgestation fetuses.

The data are tabulated to show the incidence and extent of embryonal carcinoma and control embryo cell contribution to midgestation samples. Incidence of contribution is reflected by the number of samples that contained embryonal carcinoma or control embryo cell derivatives as indicated by the presence of the Gpi-1a isozyme (# +A). In the first column, this is expressed as a fraction and percentage of the total number of samples collected and analyzed (# tot). In the second column, extent of contribution is indicated by the number of samples in which the embryonal carcinoma or control embryo cell contribution was equal to or greater than the host SWR contribution, including those instances when embryonal carcinoma or control embryo cell derivatives composed the entire fetus (# B$\geq$A). This is expressed as a fraction and percentage of the total number of samples containing any contribution by these cells (# +A).

Table 2A. Incidence and extent of contribution to midgestation fetuses: Comparison of embryo control and NG-2 aggregates.

Type of Aggregation	# +A / # tot	%	#B≥A / # +A	%
129 8-cell + 1 SWR 8-cell	4/7	57%	1/4	25%
129 ICM + 1 SWR 8-cell	15/21	71%	14/15	93%
129 emb ecto + 1 SWR 8-cell	5/17	29%	4/5	80%
NG-2 + 1 SWR 8-cell	15/19	79%	14/15	93%
small NG-2 + 1 SWR 8-cell	6/9	67%	5/6	83%
NG-2 doub/trip + 1 SWR 8-cell	2/24	8%	1/2	50%
NG-2 + 2 SWR 8-cell	15/23	65%	12/15	80%
NG-2 + 2(ICRXSWR) 8-cell	13/21	62%	2/13	15%
NG-2 + 3(ICRXSWR) 8-cell	9/11	82%	1/9	11%
NG-2 + 1(ICRXSWR) 8-cell	2/14	14%	1/2	50%

Table 2B. Incidence and extent of contribution to midgestation fetuses: Comparison of PSA-1, LT1-2D, and NG-2 aggregates.

Type of Aggregation	# +A / # tot	%	#B≥A / # +A	%
PSA-1 + 1 SWR 8-cell	19/33	58%	10/19	53%
LT1-2D + 1 SWR 8-cell	12/30	40%	9/12	75%
NG-2 + 1 SWR 8-cell	15/19	79%	14/15	93%

Table 3. Normalcy of development at midgestation of implanted aggregates

The data are tabulated to show the number of samples that developed normally in each combination (# norm), expressed as a fraction and percentage of the total number of samples in that group (# tot). The upper part of the table gives the data for all of the samples in each category of combinations and for the subset of samples that did not contain any embryonal carcinoma cell or control embryo cell derivatives (all B). The lower part of the table gives the data for the subset of samples that did contain embryonal carcinoma cell or control embryo cell derivatives as indicated by the presence of the Gpi-1a isozyme (+A). These data are further subdivided in the columns to the right into those samples in which minor contributions were present (B$>$A) and those samples in which major contributions were present (B$\geq$A).

Table 3. Normalcy of development at midgestation of implanted aggregates

Type of aggregation	# norm / # tot	%	# norm all B / # tot all B	%
129 8-cell + 1 SWR 8-cell	7/7	100%	3/3	100%
129 ICM + 1 SWR 8-cell	16/21	76%	4/6	67%
129 emb ecto + 1 SWR 8-cell	7/17	41%	6/12	50%
PSA-1 + 1 SWR 8-cell	20/33	61%	12/14	86%
LT1-2D + 1 SWR 8-cell	10/30	33%	8/18	44%
NG-2 + 1 SWR 8-cell	5/19	26%	4/4	100%
NG-2 + 1(ICRXSWR) 8-cell	12/14	86%	12/12	100%
NG-2 + 2(ICRXSWR) 8-cell	9/21	43%	5/8	63%

Type of aggregation	# norm +A / # tot +A	%	# norm B>A / # tot B>A	%	# norm B≯A / # tot B≯A	%
129 8-cell + 1 SWR 8-cell	4/4	100%	3/3	100%	1/1	100%
129 ICM + 1 SWR 8-cell	12/15	80%	1/1	100%	11/14	79%
129 emb ecto + 1 SWR 8-cell	1/5	20%	0/1	0%	1/4	25%
PSA-1 + 1 SWR 8-cell	8/19	42%	8/9	89%	0/10	0%
LT1-2D + 1 SWR 8-cell	2/12	17%	2/3	67%	0/9	0%
NG-2 + 1 SWR 8-cell	1/15	7%	1/1	100%	0/14	0%
NG-2 + 1(ICRXSWR) 8-cell	0/2	0%	0/1	0%	0/1	0%
NG-2 + 2(ICRXSWR) 8-cell	4/13	31%	4/11	36%	0/2	0%

Table 4. Implantation and development of transferred aggregates.

The number of aggregate embryos that implanted (# implant) is compared with the number of aggregate embryos that developed normally (# normal). These numbers are given as fractions and percentages of the total number transferred to females which subsequently became pregnant (# trans). In addition, the number of normal samples containing embryonal carcinoma cell or embryo control cell derivatives (#norm +A) is given as a fraction and percentage of the total number of normal samples (# normal).

Table 4. Implantation and development of transferred aggregates

Type of aggregation	# implant / # trans	%	# normal / # trans	%	# norm +A / # normal	%
mock processed 8-cell	34/120	28%	32/120	27%	------	
129 8-cell + 1 SWR 8-cell	7/19	37%	7/19	37%	4/7	57%
129 ICM + 1 SWR 8-cell	21/74	28%	16/74	22%	12/16	75%
129 emb ecto + 1 SWR 8-cell	17/101	17%	7/101	7%	1/7	14%
PSA-1 + 1 SWR 8-cell	33/98	34%	20/98	20%	8/20	40%
LT1-2D + 1 SWR 8-cell	30/115	26%	10/115	9%	2/10	20%
NG-2 + 1 SWR 8-cell	19/62	31%	5/62	8%	1/5	20%
NG-2 + 2(ICRXSWR) 8-cell	21/60	35%	9/60	15%	4/9	44%
NG-2 + 2(ICRXSWR) 8-cell (TERM)			25/190	13%	7/24	29%

Table 5. Distribution of NG-2 derivatives in the tissues of chimeric mice: Aggregation compared with blastocyst injection

The presence and relative contribution of NG-2 derivatives were estimated from GPI assays of tissue samples collected from live born mice as described in the results. The number over each column refers to the mouse from which the tissues were collected. The numbers in parentheses indicate the total number of tissues containing NG-2 derivatives in that mouse. The column on the right provides data from published blastocyst injection experiments with NG-2 for comparison. (* Each number represents % embryonal carcinoma cell contribution to that tissue in one mouse as estimated from GPI assays. From Dewey et al, 1977.)
+ $<$20%, ++ 20%-50%, +++ $\geq$50%.

Table 5. Distribution of NG-2 derivatives in the tissues of chimeric mice.

Tissue	1 (8)	2 (8)	3 (7)	4 (6)	5 (4)	6 (3)	7 (1)	blastocyst injection comparison*
1. blood		++						5
2. liver	+++	+++		++				15, 30, 5, 10
3. spleen								10, 10, 15, 5, 5
4. pancreas	+	+++		++				5, 5
5. gut (stomach)	+	+	++	++	+			40, 40, 20
6. kidney		+	++		++			50, 20, 40, 5
7. bladder	++	+	++	++				not analyzed
8. gonads	+							25, 15, 20
9. reproductive tract	+		+	++	+			20, 25, 20, 15
10. thymus			+					30, 10
11. heart	+++	+++	+++	+++	+	+++	++	55, 50, 60, 20
12. lungs	+	+	++					5, 10, 10
13. salivary glands								10
14. brain								10, 15, 10, 5, 15, 15, 10
15. muscle						+		25, 40, 20
16. pigment						+		5, 5

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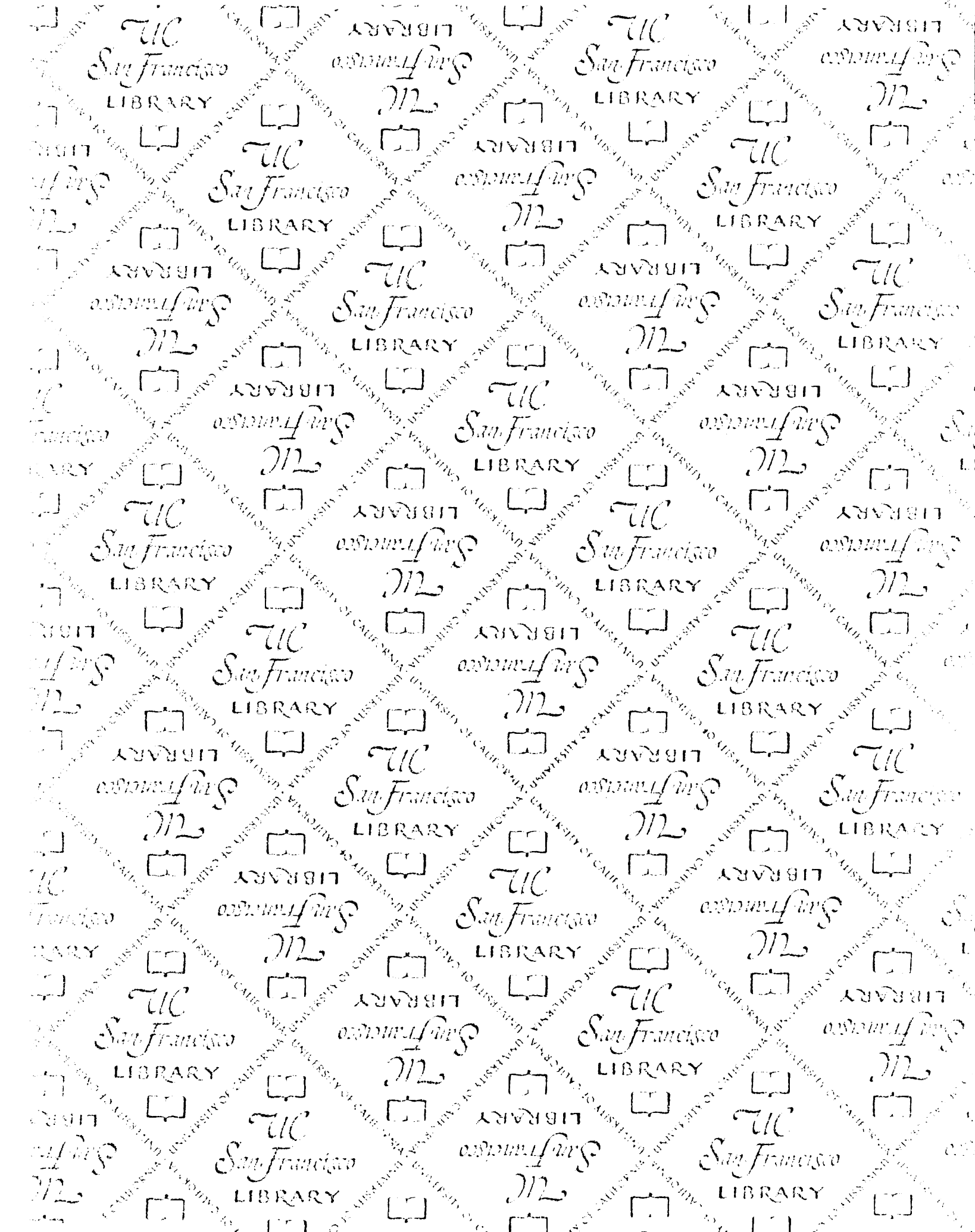
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